THE UTILIZATION OF NICOTINIC ACID BY PREGNANT WOMEN [1]

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TWO FIGURES

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A study of the incidence of pellagra in 7 cotton-mill villages in South Carolina from 1916 to 1920 (Goldberger, Wheeler and Sydenstricker, '20, '28) showed a higher incidence of pellagra among women from 25 to 45 years of age than in any other age and sex group. Kooser and Blankenhorn ('39) found a large proportion of the pellagrins among Kentucky mountain people to be multiparous women. This seems to indicate a greater demand of the maternal body for nicotinic acid during the reproductive cycle. No studies have been reported on the metabolism of this vitamin by pregnant women. The present investigation was undertaken for the purpose of studying the relation of the intake of nicotinic acid to the urinary excretion of some of its metabolites.

EXPERIMENTAL PROCEDURE

Three groups of women were selected for study. Group I [4] included 7 healthy young primigravidae during the last 5

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[4] This study is part of an investigation previously initiated for the purpose of studying the metabolism of riboflavin and thiamine by pregnant women. The riboflavin and thiamine studies were supported by a grant from the American Dairy Association through the National Dairy Council.

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months of pregnancy; group II consisted of 42 clinic patients selected at random from a group of women who were serving as subjects in a large clinical study being carried on in the Department of Obstetrics and Gynecology at the University of Chicago [5] (Dieckmann, '48); while group III included 12 non-pregnant women, graduate students in the Department of Home Economics, University of Chicago.

The 7 subjects in group I lived at home and ate self-selected diets. They were selected for study on the basis of (1) freedom from pathological disturbances which might interfere with interpretation of results, (2) willingness to cooperate and (3) accessibility to the laboratory. All except one, subject 6, were clinic patients at the Chicago Lying-in Hospital. Therefore their dietaries were no doubt influenced to some extent by instructions received from the clinic dietitian at the time of their regular clinic visits; otherwise they received no dietary instructions. Subject 6 voluntarily supplemented her diet with 11.2 mg of nicotinamide per day during the last three months of the study, while subject 7 supplemented hers with 20.2 mg of nicotinamide during the entire 5-month period.

Seven-day balance studies were made during each of the last 5 months of pregnancy, including (1) daily nicotinic acid intake, (2) urinary excretion of (a) the acid-hydrolyzable fraction and (b) N^1-methylnicotinamide.

Each subject kept a record of the weights of all foods eaten over the 7-day period, with the close supervision of one of the investigators. Individual food samples for analysis were provided by the subject in closed containers and kept under refrigeration for periods of from three to 4 days. Food composites containing one-fifth of the amount of each food consumed were homogenized in a Waring Blendor and made up to 4 l with 0.15 *N* HCl. Suitable aliquots were quantitatively transferred into an Erlenmeyer flask and autoclaved at 15 lb pressure (118–120°C.) for 20 minutes and stored in a refrigerator until analyzed.

[5] This study is being carried on in the Chicago Lying-in Hospital.

Twenty-four-hour urine collections were made over the 7-day study period in amber colored bottles containing, as preservatives, glacial acetic acid and toluene. These collections were brought to the laboratory daily and 7-day composites, containing one-tenth of the total daily amount excreted, were made for analysis.

The response to a 50 mg test dose of nicotinamide as measured by the urinary excretion of N^1-methylnicotinamide was determined at the end of each balance study. The N^1-methylnicotinamide excreted during a 4-hour collection period immediately following breakfast on the last day of the balance study served as the control level of excretion. Increased excretion of the methylated derivative after the test dose over a similar 4-hour period on the 9th day of study was used as a criterion for response to the test dose.

The data on the dietary intake of nicotinic acid of the 42 clinic subjects in group II were based upon calculated values of weighed diet records obtained under the supervision of the research dietitian of the Chicago Lying-in Hospital over periods of from 4 to 9 days. The records were carefully discussed with the subjects, and only those women whose dietary records appeared to be complete were included in the study.

The levels of excretion of N^1-methylnicotinamide of this group of subjects were determined by assay of a composite of three 24-hour urine collections — the period of collection corresponding to the last three days of the dietary study. Creatinine excretion [6] was determined on each 24-hour collection. A daily excretion of 0.95 gm or above of creatinine was considered indicative of complete urine collections.

Concurrent with the study of the two groups of pregnant women, some information on the dietary intake, urinary excretion of N^1-methylnicotinamide and response to a 50 mg test dose of nicotinamide was obtained on 12 non-pregnant women. These data are included here for the purpose of comparing the pattern of excretion of the metabolic products of nicotinic acid

[6] The authors were very kindly given access to the results of creatinine determinations which were being made in the laboratories of the Chicago Lying-in Hospital.

by pregant and non-pregnant women. The data on the dietary intake of nicotinic acid of the 12 women in group III were based upon calculated values of two week-day records of food intake estimated in grams. Twenty-four-hour urine collections were made the second day of the dietary study. The response to a 50 mg test dose of nicotinamide on the third day was determined for this group of subjects according to the procedure described above for group I.

The N^1-methylnicotinamide in urine was determined by the acetone-fluorometric method of Huff and Perlzweig ('47). Digests of the diet and urine composites, made with 2 *N* H_2SO_4, were assayed for nicotinic acid by the chemical method (cyanogen bromide-amine reaction) as modified by one of the present authors (Frazier, '47). Aliquots of the digest of diet composites were extracted with chloroform before assay, since fat was found to interfere with the chemical assay. The acid digest of the urine included free nicotinic acid, hydrolyzed nicotinamide and any nicotinuric acid present.

RESULTS

The relation of the dietary intake of nicotinic acid to the urinary excretion of some of its metabolic products

Group I. The daily intake of nicotinic acid and the urinary excretion of the acid-hydrolyzable fraction and N^1-methylnicotinamide, as well as the percentage recovery of a 50 mg test dose of nicotinamide, for the 7 primigravidae during each of the 5 7-day study periods are presented in table 1. There was considerable variation from month to month in the intake of nicotinic acid by individual subjects. The average daily intake for the 5 subjects on unsupplemented dietaries ranged from 13.8 to 18.3 mg. The average daily intake for all subjects was 19.7 mg.

The average daily excretion of the acid-hydrolyzable metabolites of nicotinic acid was 1.1 mg. The level of excretion of

TABLE 1

The nicotinic acid intake, urinary excretion of the acid-hydrolyzable fraction and N^1-methylnicotinamide, and per cent recovery of a 50 mg test dose of nicotinamide by 7 primigravidae from the 5th through the 9th months

SUBJECT	STUDY PERIOD	INTAKE NICOTINIC ACID	EXCRETION, EXPRESSED AS NICOTINIC ACID				RECOVERY OF TEST DOSE
			Acid hydrolyzable	N^1-methylnicotinamide	Total		
					Amount	Fraction of intake	
	mo.	mg	mg	mg	mg	%	%
1	5	16.6	1.7	7.3	9.0	54	15.6
	6	16.9	1.4	6.8	8.2	48	15.1
	7	18.7	1.0	8.8	9.8	52	21.0
	8	15.5	1.4	13.0	14.4	93	20.0
	9	15.0	1.4	13.1	14.5	97	23.3
	Average	16.6	1.4	9.8	11.2	67	19.1
2	5	16.2	1.0	9.1	10.1	62	15.8
	6	16.6	0.5	9.0	9.5	51	14.1
	7	12.2	0.7	12.4	13.1	107	18.0
	8	9.8	0.8	8.1	8.9	91	28.3
	9	14.4	0.9	17.7	18.6	128	24.4
	Average	13.8	0.7	11.3	12.0	87	20.1
3	5	22.9	1.0	10.3	11.3	49	19.0
	6	16.4	0.7	9.9	10.6	64	8.0
	7	20.9	1.3	14.0	15.3	73	19.8
	8	17.2	1.1	15.1	16.2	94	10.1
	9	14.3	0.9	13.9	14.8	102	13.1
	Average	18.3	1.0	12.6	13.6	74	14.0
4	5	12.9	0.8	10.8	11.6	90	16.2
	6	17.2	1.0	10.7	11.7	68	15.1
	7	21.6	0.7	14.0	14.7	68	19.5
	8	18.3	1.1	16.9	18.0	98	22.9
	9	16.6	1.7	15.8	17.5	106	13.8
	Average	17.3	1.0	13.6	14.6	83	17.1
5	5	18.3	1.3	10.3	11.6	63	12.0
	6	20.6	1.2	15.1	16.3	80	16.7
	7	11.8	1.3	13.5	14.8	125	20.3
	8	12.6	0.9	13.5	14.4	114	19.5
	9	20.2	1.6	10.6	12.2	60	17.2
	Average	16.7	1.3	12.0	13.5	80	17.1
6	5	18.6	0.7	10.5	11.2	60	23.8
Supplement	6	11.8	1.3	9.8	11.1	94	8.4
11.2 mg	7	24.6	1.1	14.5	15.6	63	23.8
per day 7,	8	28.4	0.9	14.8	15.7	55	16.7
8, 9 mos.	9	24.9	0.9	15.6	16.5	66	17.5
	Average	21.7	1.0	13.0	14.0	64	18.0
7	5	31.5	2.2	23.7	25.9	82	23.2
Supplement	6	37.1	1.0	22.7	23.7	64	25.9
20.2 mg per	7	33.6	1.2	22.2	23.4	69	24.5
day all	8	34.0	2.1	23.1	25.2	74	32.2
periods	9	32.8	1.3	14.8	16.1	49	...
	Average	33.8	1.6	21.3	22.9	68	26.5
All	Average	19.7	1.1	13.8	14.9	75	18.8

this portion of the urinary components remained fairly constant at from 5 to 8% of the intake.

The largest proportion of the metabolites studied was in the form of the methylated derivative. The average daily excre-

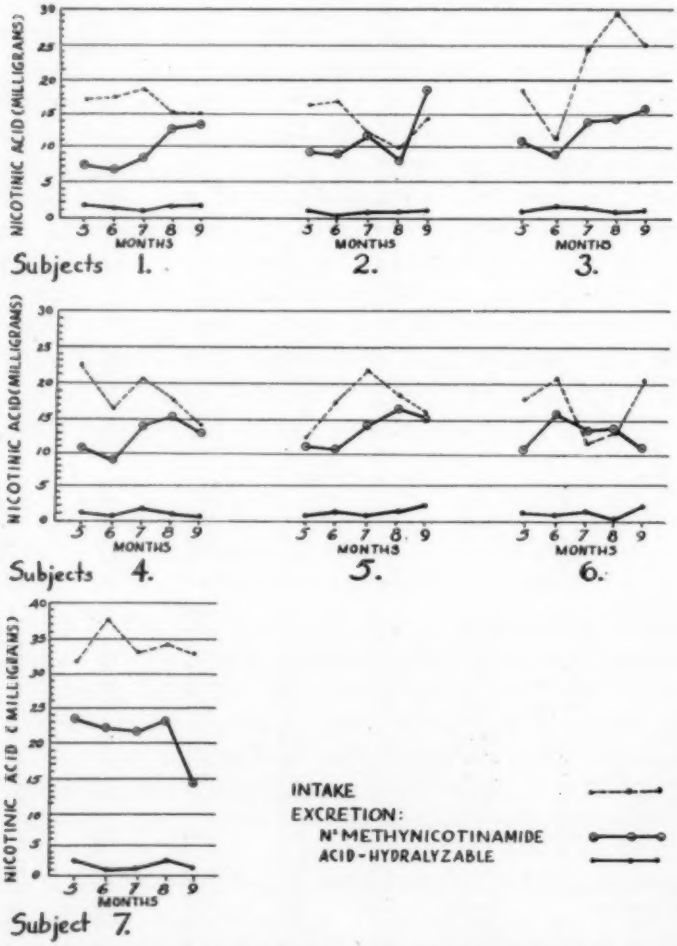

Fig. 1 The daily intake of nicotinic acid, and urinary excretion of the acid-hydrolyzable fraction and N^1-methylnicotinamide (expressed as nicotinic acid) of 7 primigravidae.

tion of N^1-methylnicotinamide for all subjects over the 5-month period was 13.8 mg (expressed as nicotinic acid). The amount of N^1-methylnicotinamide excreted by the 5 subjects on unsupplemented dietaries showed only slight relation to intake. Although there was some variation between study periods of individual subjects, in general there was a definite increase in excretion of the methylated derivative, irrespective of intake, with increased duration of pregnancy. For example, the excretion of N^1-methylnicotinamide (expressed as nicotinic acid) of subject 1 increased from 7.3 to 13.1 mg during the 5-month study period. This resulted in increasing the excretion of the total metabolites studied from 54 to 97% of the intake.

The two subjects who received a daily supplement of nicotinamide (subjects 6 and 7, table 1) failed to show similar increases in excretion of the methylated derivative but showed, rather, a more consistent and, in the case of subject 7, a higher level of excretion. Although the total amounts of metabolites excreted by these subjects were more than those excreted by the 5 subjects on the lower unsupplemented intakes, the percentage of intake excreted was less.

Figure 1 presents graphically the relation of intake of nicotinic acid to the urinary excretion of both the acid hydrolyzable fraction and N^1-methylnicotinamide for each of the 7 subjects during the 5 study periods. Here the variation in intake of the subjects on the unsupplemented dietaries, the constant level of excretion of the acid-hydrolyzable fraction, and the increase in excretion of N^1-methylnicotinamide during the last trimester become apparent.

Table 2 (part A) summarizes average intakes and excretions for all subjects in group I according to months of pregnancy. The average intake per month remained quite constant, as is true also of the average excretion of the acid-hydrolyzable fraction. The average excretion of N^1-methylnicotinamide, however, increased from 11.7 to 14.5 mg (nicotinic acid), representing an increase in excretion from 60 to 74% of intake.

TABLE 2

A summary of the average daily intake of nicotinic acid and urinary excretion of some of its metabolites by primigravidae

MONTH OF PREGNANCY	NUMBER OF SUBJECTS	INTAKE NICOTINIC ACID	EXCRETION				FRACTION OF INTAKE EXCRETED		
			Acid hydrolyzable	N^1-methylnicotinamide	Nicotinic acid equiv.	Total	Nicotinic acid	N^1-methylnicotinamide	Total
		mg	*mg*	*mg*	*mg*	*mg*	%	%	%
A. Subjects on both unsupplemented and supplemented dietary intakes									
5	7	19.6	1.2	13.0	11.7	12.9	6	60	66
6	7	19.5	1.0	13.3	12.0	13.0	5	62	67
7	7	20.5	1.0	15.8	14.2	15.2	5	69	74
8	7	19.4	1.2	16.6	14.9	16.1	6	77	83
9	7	19.7	1.2	16.1	14.5	15.7	6	74	80
B. Subjects on unsupplemented dietary intakes									
5	6	17.6	1.1	10.8	9.7	10.7	6	57	63
6	6	16.6	1.0	10.2	10.2	11.2	6	61	67
7	5	17.0	1.0	14.1	12.5	13.5	6	72	80
8	5	14.7	1.1	14.8	13.3	14.4	7	91	98
9	5	16.1	1.3	15.8	14.2	15.5	8	90	98

A statistical covariance analysis [7] of these data showed that with the elimination of the influence of intake the variation in excretion of the total metabolic products studied was not significant. However, this analysis *did* show significant differences among the patterns of excretion of individual subjects. Accordingly, a covariance analysis of total excretion (Y) on intake (X) for the 5 subjects on unsupplemented intakes with similar patterns of urinary excretion (table 2, part B) showed no significant differences among the subjects.

TABLE 3

The relation of dietary intake of nicotinic acid to the urinary excretion of N^1-methylnicotinamide by 42 pregnant women at Chicago Lying-in Hospital clinics

TRIMESTER OF PREGNANCY	NO. OF SUBJECTS	AVERAGE DAILY INTAKE NICOTINIC ACID	AVERAGE DAILY EXCRETION	
			N^1-methyl nicotinamide (nicotinic acid equiv.)	Fraction of intake
		mg	*mg*	%
1	5	14.8	11.2	75
2	19	12.4	10.9	80
3	18	11.1	13.1	118
	42	12.1	11.9	98

A test of significance between the adjusted means for each of the 5 months showed, however, a significant difference in total excretion during the last trimester at the 5% probability level.

Confidence limits for population months of pregnancy indicated no significant difference in total excretion by the subjects between the two months studied in the second trimester, or between the three months in the third trimester.

Thus, there is statistical evidence that a real difference exists in the levels of excretion of N^1-methylnicotinamide by primigravidae on unsupplemented dietary intakes of nicotinic acid with progression of pregnancy. Moreover, the statistical analysis verifies the observation that the pattern of excretion

[7] The authors are indebted to Dr. T. A. Bancroft, Director of the Statistics Laboratory, Alabama Polytechnic Institute, for statistical analysis of these data.

of the two subjects on the supplemented intakes was significantly different from that of the 5 subjects on unsupplemented dietary intakes.

Group II. Table 3 presents the data on the average daily intake of nicotinic acid, excretion of N^1-methylnicotinamide and percentage of intake of this metabolite excreted, for 42 clinic subjects grouped according to trimester of pregnancy.

There was considerable variation in dietary levels of nicotinic acid within this group. The total average calculated intake was 12.1 mg, which represented a range of from 6.4 to 24.5 mg per day. In table 4 the subjects have been grouped

TABLE 4

The relation of dietary intake of nicotinic acid to urinary excretion of N^1-methylnicotinamide by 42 patients of Chicago Lying-in Hospital clinics

NUMBER OF SUBJECTS	INTAKE		EXCRETION		FRACTION OF INTAKE EXCRETED
	Average	Range	N^1-methylnicotinamide	Nicotinic acid equivalents	
	mg	*mg*	*mg*	*mg*	%
4	6.4	4.6–7.4	12.4	11.2	175
11	8.7	7.5–9.9	12.1	10.9	125
12	11.3	10.0–12.4	12.7	11.4	100
6	13.4	12.5–14.9	13.4	12.1	90
9	18.4	15.0–24.5	14.1	12.7	62
42	12.1		13.1	11.9	98

according to range of intake as follows: Those who received 4.6–7.4; 7.5–9.9; 10.0–12.4; 12.5–14.9; and 15.0–24.5 mg per day. On intakes within these ranges the average amounts of N^1-methylnicotinamide excreted (expressed as nicotinic acid) were 11.2, 10.9, 11.4, 12.1 and 12.7 mg, respectively. These data indicate no apparent relation between intake of nicotinic acid and excretion of N^1-methylnicotinamide; rather, they indicate a more or less constant level of excretion of the methylated derivative regardless of intake (fig. 2).

The data for this group of subjects confirm the observations on the subjects in group I in two important respects. First,

the 9 subjects in group II with an average intake of 18.4 mg excreted an average of 14.1 mg of N^1-methylnicotinamide per day (table 4) while the 7 subjects in group I on a comparable intake of 19.7 mg excreted an average of 13.8 mg of this metabolite (table 1). Second, there was also an increase in level of excretion of N^1-methylnicotinamide by this group with increased duration of pregnancy, as in the case of the 5 primigravidae in group I on unsupplemented intakes. The average percentages of intake excreted were 75, 80, and 118 for the first, second and third trimester, respectively (table 3). Fourteen of the clinic subjects were primigravidae, 7 in each of the

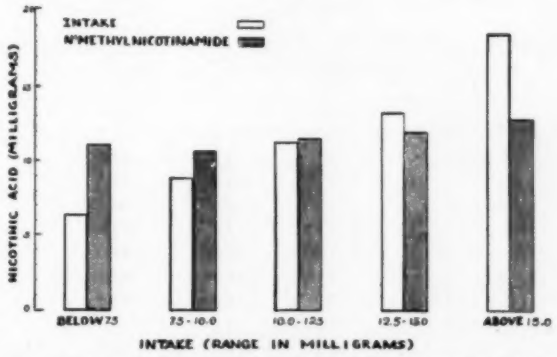

Fig. 2 The relation of dietary intake of nicotinic acid to the urinary excretion of N^1-methylnicotinamide (expressed as nicotinic acid) of pregnant women.

second and third trimesters. This group is comparable, therefore, to the 5 subjects in group I with respect to gravida and trimester of pregnancy; it differs, however, in precision of measurement, since the excretion values on the 5 subjects in group I were means of 5 study periods of 7 days each, while the 14 observations on subjects in group II represent single study periods of three days each. A statistical covariance analysis of excretion (Y) on intake (X) showed no significant difference in performance between the two groups when a test of significance of the adjusted excretion means was made.

Group III. The data on the nicotinic acid intake, excretion of N^1-methylnicotinamide, and the percentage recovery of a 50 mg test dose of nicotinamide over a 4-hour period for the 12 non-pregnant women are shown in table 5.

There was a considerable range in intake, from 8.6 to 15.9 mg, with an average for all subjects of 12.7 mg per day.

TABLE 5

The dietary intake of nicotinic acid (calculated) and the urinary excretion of N^1-methylnicotinamide by non-pregnant women

SUBJECT	INTAKE PER DAY	EXCRETION			FRACTION OF 50 MG NICOTINAMIDE
		N^1-methyl-nicotinamide	Nicotinic acid equivalent	Fraction of intake	
	mg	*mg*	*mg*	%	%
A	14.1	4.0	3.6	25.5	9.1
B	14.5	3.4	3.1	21.3	13.8 [1]
C	13.0	3.3	2.9	22.3	3.0
D	13.2	5.1	4.6	42.4	4.6
E	11.1	4.9	4.4	39.6	9.0
F	10.2	9.7	8.7	85.3	7.1
G	8.6	5.3	4.8	55.8	11.5
H	15.9	7.8	7.0	44.0	6.7
I	15.7	5.2	4.7	30.0	7.0
J	10.9	8.7	7.8	72.2	11.2
K	13.0	7.2	6.5	50.0	10.4
L	12.4	7.4	6.7	54.0	8.9
Average	12.7	6.0	5.4	42.5	8.4 [2]

[1] Seventy-five milligram test dose.
[2] Exclusive of subject B.

Again, there was variation among subjects in the amount of N^1-methylnicotinamide excreted irrespective of intake. For instance, subject B on an intake of 14.5 mg of nicotinic acid excreted 3.1 mg of N^1-methylnicotinamide (expressed as nicotinic acid) or 21.3% of the intake. Subject F, on the other hand, excreted 8.7 mg, or 85.3% of an intake of 10.2 mg.

The level of excretion of the methylated derivative, however, was much lower than was found in the case of the preg-

nant women. The average daily excretion of this compound was 6.0 mg, or 5.4 mg nicotinic acid equivalents. The average percentage of intake excreted by this group of subjects was 42.5.

Response to a test dose of nicotinamide

The data on the percentage recovery of the 50 mg test dose of nicotinamide over the 4-hour period for the 7 primigravidae in group I, and for the 12 non-pregnant women in group III, are shown in tables 1 and 5, respectively. There was some variation in response to the test dose among subjects within

TABLE 6

The relation of dietary intake of nicotinic acid to the average percentage return of a 50 mg test dose of nicotinamide by primigravidae

SUBJECT BY NUMBER	INTAKE OF NICOTINIC ACID	RETURN OF 50 MG OF NICOTINAMIDE
	mg	%
2	13.8	20.1
1	16.6	19.1
5	16.7	17.1
4	17.3	17.1
3	18.3	14.0

the groups, as well as among study periods of individual subjects in group I.

In general there was a slight tendency toward an increase in percentage return by the pregnant women from the 5th through the 9th month. Again the performance of subject 7 differed from that of the subjects on the lower unsupplemented dietary intakes. Although the data for this subject are incomplete due to the fact that she delivered before the completion of the last study period, the percentage recovery of the test dose was consistently higher than that of the other subjects and was fairly constant.

Both groups I and III showed a small but consistent decrease in the percentage return of the 50 mg of nicotinamide

with increased levels of intake. The relation of dietary intake to average percentage return of the test dose is shown in table 6, which lists the 5 subjects in group I on unsupplemented dietary intakes in ascending order according to average intake of nicotinic acid. Although the dietary data on the non-pregnant women were less precise, a similar trend in decreased percentage return of the test dose with increased intake of nicotinic acid was observed among these subjects (table 5).

DISCUSSION

The function of the coenzymes in carbohydrate metabolism suggests a direct relationship between the production of energy and the utilization of nicotinic acid by the body. Sandiford, Wheeler and Boothby ('30) found that total heat production during pregnancy increased gradually from the end of the third month. During later pregnancy the rate increased more rapidly, and reached its highest point just before delivery. The fact that the basal metabolic rate immediately following delivery was comparable with the average rate during the first lunar month, and that the increased rate during late pregnancy could be accounted for by added metabolism of fetus, placenta, and accessory structures, led these investigators to conclude that there was probably no change in actual rate or intensity of heat production per unit mass of maternal tissue. This seems to suggest that the observed increase in level of excretion of N^1-methylnicotinamide during the last trimester of pregnancy might be attributed to an increase in the requirement of the maternal organism for coenzymes I and II paralleling the increase in total body metabolism. The conclusions of Sandiford et al. further suggest a participation of the fetal liver in the total methylation of the pyridine compound during prenatal life.

This reasoning is based upon the assumption that the methylated derivative is a true metabolite of nicotinic acid, i.e., that it is an end product of intermediary metabolism and that an increased excretion of N^1-methylnicotinamide is indicative of an increased rate of turnover of the coenzymes.

The increase in percentage return of a test dose of nicotinamide by the subjects in groups I and III with decreased levels of dietary intake supports this hypothesis.

Thus it is conceivable that with the increased demands of the maternal organism more of the vitamin is mobilized to meet body needs, which results in an increased excretion of its metabolites. This interpretation of the pattern of urinary excretion of the methylated derivative of this vitamin is directly opposite to the usual interpretation of an increased excretion of the water soluble vitamins following test doses; namely, that the higher the quantity of the test dose returned the lower the quantity of the test dose utilized. Clarification of this problem must await additional information on the level of excretion of the metabolite of nicotinamide, N^1-methyl-6-pyridone-3-carboxylamide, first described by Knox and Grossman ('47), as well as on the interrelation of tryptophan and nicotinic acid on known levels of intake.

An examination of the food intakes of the subjects in group I revealed that the level of protein consumed by each subject was within normal limits as to quantity; was derived largely from milk and milk products, meat, eggs, and cereals; and did not *significantly* change either in character or amount throughout the 5-month period of study. Actually there was a slight decrease in daily intake of protein (8–10 gm) in the 9th month as compared to the 5th month in 6 of the 7 subjects in this group. This slight decrease in protein intake may have been a reflection of a seasonal difference in the types of foods prepared and selected by the subjects in their winter and summer diets, since most of the subjects delivered in the summer. Although no quantitative data on tryptophan intakes were determined from these diets, it would appear unlikely that tryptophan intakes increased as pregnancy progressed.

The constant levels of excretion of N^1-methylnicotinamide by subjects 6 and 7, and the higher excretion by subject 7, on supplemented intakes of nicotinamide indicate that, with the ingestion of a constant relatively high amount of the amide over a period of time, there is a turnover of coenzymes suf-

ficient to meet, or even in excess of, the increased demand during later pregnancy. The uniform response of subject 7 to the test dose also reflects this concept. The failure of these two subjects to show an increased excretion of N^1-methylnicotinamide during the last trimester suggests that an intake of 20 mg or above of nicotinic acid is in excess of the daily requirements during pregnancy.

The average 24-hour excretion of 5.4 mg equivalents of nicotinic acid as the methylated derivative by the normal controls in group III approximates that reported by other investigators for healthy adult women on average diets. On intakes of approximately 10 mg Ellinger and Benesch ('45) report an average daily excretion of 3.8 mg of nicotinic acid equivalents by 5 adult women, as measured by a modification of the method described by Najjar ('44). Employing a modification of this method, Coryell et al. ('47) found an average excretion of 3.4 mg equivalents of N^1-methylnicotinamide (corrected for the 19% error) by 5 women during the lactation period of from 68 to 306 days *post partum*. An examination of these data shows a slight but consistent increase in excretion of N^1-methylnicotinamide during late lactation, an approach to the values obtained for normal adult women. Perlzweig and associates ('47) found an average excretion of 5.9 mg of nicotinic acid equivalents by three adult women on "good average diets." These data were obtained by the method of assay employed in the present study (Huff and Perlzweig, '47) and agree with the average excretion for the normal controls in group III of 5.4 mg.

The constant level of excretion of the acid-hydrolyzable nicotinic acid of from 5 to 8% of the intake (table 2) is consistent with that observed by other investigators (Sarett, Huff and Perlzweig, '42; Johnson, Hamilton and Mitchell, '45; Oldham, Davis and Roberts, '46), all of whom found similar levels of excretion of the acid-hydrolyzable fractions regardless of the levels of intake. Such a level of excretion may represent the renal threshold for this group of compounds — namely, nicotinic acid, nicotinamide, and nicotinuric acid.

SUMMARY

1. The dietary intake of nicotinic acid, the urinary excretion of the acid-hydrolyzable fraction and N^1-methylnicotinamide, and the response to a 50 mg test dose of nicotinamide, were studied on 7 primigravidae during each of the last 5 months of pregnancy. In addition, a study was made of the dietary intake of nicotinic acid and the urinary excretion of N^1-methylnicotinamide by 42 clinic subjects. The estimated intake of nicotinic acid, the urinary excretion of N^1-methylnicotinamide and the response to a 50 mg test dose of nicotinamide were observed on 12 non-pregnant women.

2. There was a constant level of excretion of the acid-hydrolyzable fraction regardless of intake.

3. There was a statistically significant increase in the excretion of N^1-methylnicotinamide by the 5 primigravidae in group I on unsupplemented diets during the last trimester of pregnancy. This observation was confirmed by the data on the 14 primigravidae in group II.

4. The significance is discussed of the relation of the intake of nicotinic acid to the urinary excretion of N^1-methylnicotinamide and the percentage return of a 50 mg test dose of nicotinamide.

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THE DETERMINATION OF EARLY THIAMINE-DEFICIENT STATES BY ESTIMATION OF BLOOD LACTIC AND PYRUVIC ACIDS AFTER GLUCOSE ADMINISTRATION AND EXERCISE [1]

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FIVE FIGURES

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It is important to develop a method which will evaluate metabolic insufficiency prior to the development of the more obvious clinical signs of thiamine deficiency. Low levels of thiamine in blood and urine give valuable information about the recent dietary history of the patient, but a method which identifies the disturbance of cell metabolism resulting from these dietary inadequacies facilitates more specific interpretations.

Friedemann and Barborka ('41) have shown that there is a definite ratio between levels of lactate and pyruvate in the blood. Stotz and Bessey ('42), working with pigeons, advocated the use of blood lactate-pyruvate ratios to distinguish true abnormality of pyruvate metabolism from the fluctuations due to exercise, food consumption and anoxia. As a result of the emphasis placed upon increases in pyruvic acid associated with thiamine deficiency there have been many clinical attempts to use the basal level of pyruvic acid in the blood as an indica-

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tion of the degree of thiamine deficiency of patients. On the whole there has been little agreement between values of blood pyruvic acid and the degree of early thiamine deficiency states (Keys, Henschel, Taylor, Mickelsen and Brozek, '45; Berryman et al., '47). This discrepancy may be due either to the variability of the basal level of the pyruvic acid or to the lack of agreement regarding the sub-clinical signs of thiamine deficiency, or to both reasons.

Bueding, Stein and Wortis ('40) showed that the ingestion of glucose caused a temporary but significant elevation of blood pyruvic acid, which was abnormally prolonged and elevated in thiamine deficiency. Williams, Mason, Smith and Wilder ('40) made similar observations correlating the levels of the lactic and pyruvic acids after glucose administration during experimentally produced thiamine deficiencies. The opportunities provided by a long-term controlled study in thiamine deficiency (Horwitt, Liebert, Kreisler and Wittman, '48) made it possible to test published procedures for evaluating thiamine deficiency and to develop a method which determines the combined effects of glucose ingestion and mild exercise on the blood lactic and pyruvic acids.

METHODS

Glucose was estimated by the procedure described by Hoffman ('37). The *pyruvic acid* and *lactic acid* methods used were those reported by Friedemann and Haugen ('43) and Barker and Summerson ('41), respectively. Modifications in these methods designed to insure reproducibilty of results and special precautions employed in the handling of blood have been described elsewhere (Horwitt et al., '48). *Oral glucose* was administered immediately after withdrawal of the fasting basal blood sample (9 ml 20% glucose per kilogram). *The mild exercise test*, which was applied 60 minutes after glucose ingestion, consisted of walking down and up, and then down and up again, a flight of 21 steps, each step being 19 cm high. The rate of descent and climb was controlled so that the exercise was completed in 60 seconds. The lactic and pyruvic acids reach

their peak about 5 minutes after exercise; therefore the blood drawn at that time is called the 66 minute sample.

The details of the *human thiamine depletion study* and the elaborate dietary and analytical precautions taken to insure accuracy of food intake have been described elsewhere (Horwitt, Liebert, Kreisler and Wittman, '48). In summary, 36 male patients were selected from the State Hospital population and divided into three groups, A, B and C. Each person in the A group received a daily diet containing approximately 2,200 calories, which was apparently adequate in all the essentials except thiamine, of which approximately 400 μg were present, and riboflavin, of which the diet contained about 900 μg. Those in the B group received the same diet during the first two years of the experiment except that a daily supplement of yeast extract was given, containing approximately 6 mg of thiamine and 1.3 mg of riboflavin. The members of the C group received the regular hospital diet, which they ate ad libitum. Dietary checks of their food consumption indicated that they ingested approximately 1 mg of thiamine per day.

The clinical effect of the restriction of thiamine to 400 μg per day in the A group was at no time more than minimum. Therefore, after 25 months of this regimen, it was decided to feed the B group a diet containing approximately half as much thiamine as the A group were receiving. Accordingly the supplementation of the B group was stopped and the subjects of this group were placed on a diet which provided approximately 200 μg of thiamine and 800 μg of riboflavin daily.

During the more than three years in which these subjects were observed, studies were made on the blood glucose, lactic and pyruvic acid levels basally, after glucose administration and after glucose followed by exercise. The results were correlated with the diets fed and with the clinical signs of thiamine deficiency which appeared.

RESULTS

The *basal blood levels of lactic and pyruvic acid* did not correlate well with the states of thiamine deprivation. The 5 young

men in the B group, after two months on 200 μg of thiamine daily, had levels of 1.05, 1.05, 1.07, 0.82, and 1.05 mg % for pyruvic acid, respectively, which were similar to those they had shown for the previous two years. After 4 months on this diet, when most of them showed load-test evidence of abnormality, they had levels of 0.96, 1.24, 1.22, 1.06, and 1.39 mg %, respectively. The old men in the B group had control basal levels of 0.86, 0.68, 0.96, 0.66 and 0.98 mg %, respectively, and after two months on the restricted diet, levels of 0.80, 1.12, 0.76, 1.29, 1.03 and 0.97. Only after 4 months, when the load-test results were all abnormally high, were the basal pyruvate levels somewhat higher, being 1.18, 1.50, 1.36, 1.10 and 1.20 mg % (1 lost). While there was this trend toward higher basal levels with continued dietary restriction, the amount of the increase was not significant in most cases. Experience with several hundred individuals on apparently normal diets has indicated that the basal pyruvic acid levels show large variations that are not consistent with levels obtained at 60, 66 and 120 minutes after glucose ingestion. Though it is proper to suspect pathology whenever a basal pyruvic acid level exceeds 1.20 mg %, such levels are not uncommon, even in the absence of any apparent metabolic disorders. It is significant, however, that the individual (BO2) who showed the most advanced neurological pathology had the two highest basal levels of pyruvic acid, 1.50 and 1.98 mg %, the latter taken at the height of his clinical pathology.

The basal lactic acid data showed an approximate 10 to 1 correlation with pyruvic acid. Thus, on the day that the basal pyruvic acid levels for the old subjects of the B group were 1.18, 1.50, 1.36, 1.10 and 1.20 mg %, the lactic acid values were 11.5, 15.6, 11.9, 11.1 and 11.3 mg %, respectively.

Glucose as a "metabolic load"

The changes in blood levels of lactic and pyruvic acid after glucose administration were determined bimonthly. Data obtained at 60 and 90 minutes after glucose administration have

been recorded previously (Horwitt et al., '48). These data indicated that though the average results with the depleted subjects were somewhat higher than those with subjects on an adequate thiamine intake, the individual figures overlapped. The averages, while significant for a group, were not indicative of a test sufficiently sensitive to permit the detection of early abnormality in the individual.

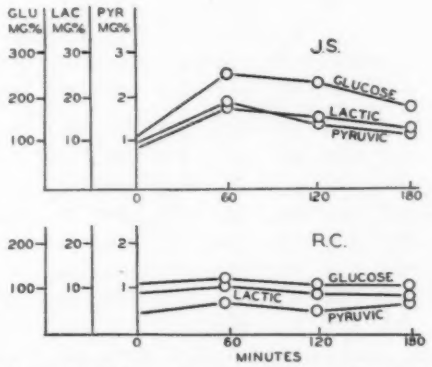

Fig. 1 Comparison of relative changes in lactic and pyruvic acid with high and low glucose tolerance curves.

Glucose plus exercise as a "metabolic load"

Mild exercise alone as a "metabolic load" showed promise of distinguishing deficient from non-deficient subjects. This criterion was abandoned, however, because the exercise test used produced only a small rise in lactic and pyruvic acids and the unreliable base line made such a rise difficult to interpret. Severe exercise tests could not be used since they provide data which are too dependent upon cooperation, training and cardiovascular efficiency. The near success of the mild exercise test prompted the next step, namely, to combine glucose ingestion and mild exercise to form a double "metabolic load." It was reasoned that this would provide a greater strain on the mechanisms of carbohydrate metabolism and also make it possible

to utilize the more accurate 60 minute base line. The results of this glucose-exercise test (see Horwitt et al., '48) showed a more marked difference among the experimental groups than appeared with glucose alone, but individual variations gave evidence that the glucose levels should be considered in order to avoid misinterpretations of the data.

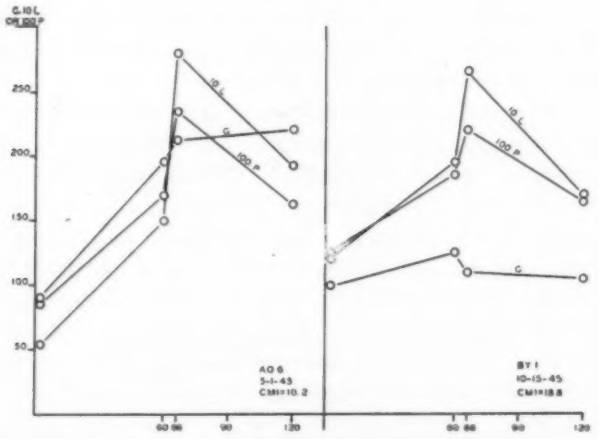

Fig. 2 Comparison of glucose, lactate and pyruvate results obtained from subject AO6, who showed no positive signs of clinical thiamine deficiency at the time of test, and subject BY1 when the latter was showing definite signs of thiamine depletion. Lactic and pyruvic acid results are multiplied by 10 and 100, respectively, to bring them to the same scale as glucose.

Correlation of blood glucose with blood lactate and pyruvate

The importance of this step is more apparent if the lactic and pyruvic acid levels of patients with hypoglycemic and hyperglycemic glucose tolerance curves are compared. Figure 1 presents an example of observations on two such individuals, both of whom were receiving adequate amounts of thiamine. The subject, J. S., with a curve illustrative of alimentary hyperglycemia, had high levels of lactic and pyruvic acid by usually accepted standards, but he also had glucose levels which were high. Conversely, subject R. C., with a flat glucose

tolerance curve, had correspondingly low lactic and pyruvic acid levels. Figure 2 presents data from two subjects on thiamine-deficient diets. One, BY1, was consuming the diet containing 200 μg of thiamine daily; the other, AO6, the diet with 400 μg. The former showed definite signs of clinical thiamine deficiency; the latter, although having equally high

TABLE 1

Relationship of glucose and lactic and pyruvic acid before and after exercise[1]

TIME	BASAL			60 MINUTES			66 MINUTES			120 MINUTES		
Subject	Gluc.	Lact.	Pyr.	Gluc.	Lact.	Pyr.	Gluc.	Lact.	Pyr.	Gluc.	Lact.	Pyr.
AY3	118	9.0	1.14	231	20.5	2.12	188	30.5	2.54	114	19.3	1.76
AY4	110	11.5	0.90	260	25.6	2.32	168	31.1	2.28	154	20.6	1.08
BO5	107	8.4	1.08	233	16.5	1.38	199	22.3	1.33	196	15.4	1.23
BY5	104	9.0	1.08	170	16.6	1.52	135	20.2	1.30	125	12.9	1.10
T. M.	121	6.9	0.69	218	20.1	2.00	176	19.8	1.53	137	13.8	1.50
J. S.	106	9.7	1.23	195	15.4	1.56	173	18.2	1.57	146	11.5	1.12
E. D.	120	8.6	1.00	206	12.5	1.05	175	22.0	1.43	158	9.4	0.72
F. D.	105	6.6	0.85	197	13.0	1.04	155	17.5	1.50	125	12.5	1.14

[1] One minute of stair climbing was instituted 60 minutes after the ingestion of glucose. The 66 minute samples were obtained 5 minutes after the completion of this exercise. These data are selected to show how the ratio is affected in situations where there is an unusual decrease of blood glucose following exercise, sometimes necessitating a decrease in pyruvic acid. This demonstrates the tendency toward maintenance of the lactate to pyruvate post-exercise ratio of approximately 15 to 1.

levels of lactic and pyruvic acid, showed no clinical signs of deficiency. However, when the levels of glucose were taken into consideration, it became apparent that AO6 was functioning at a higher blood sugar level and therefore was under a greater "metabolic load" at the time of the test.

Comparison of lactic and pyruvic acid levels of different individuals can only be made if they have similar levels of glucose. In other words, a high lactate or pyruvate figure should not be considered pathological unless it has been compared with the blood glucose level obtained at the same time,

due to the fact that the latter level is indicative of a greater or lesser load being applied to the enzyme systems involved.

Individuals in a satisfactory state of alimentation, when given the glucose-plus-exercise test, had levels of lactic acid 5 minutes after the exercise which were approximately one-tenth their levels of glucose, and levels of pyruvic acid which were approximately one-fifteenth their levels of lactic acid. When the increase of lactate after applied exercise is minimum, the increase of pyruvate is small or absent, thereby maintaining the approximate ratio of 1 to 15. This hypothesis was tested in those cases in which the glucose level showed a greater than average decrease after exercise. The data given in table 1 were chosen from all the experiments conducted during the past 4 years and represent atypical decreases in blood glucose obtained after exercise. By comparing the lactic and pyruvic figures at 60 minutes with those at 66 minutes (table 1), one may note how often the pyruvic acid does not increase following exercise, thus maintaining the ratio of approximately 1 to 15. Usually the glucose does not decrease appreciably after exercise and there is a greater increase in lactic acid which is in turn accompanied by a more definite rise in pyruvic acid, thus maintaining this approximate ratio.

The index of carbohydrate metabolism

Inasmuch as differences due to variations in glucose tolerance appeared quite frequently, a simple arithmetical expression was developed to relate the values of lactic and pyruvic acid to those of glucose. The formula is based on analysis of tests on several hundred different subjects. In each instance the blood levels for glucose, lactic acid and pyruvic acid were those obtained 5 minutes after the completion of the mild stair-climbing test applied 60 minutes after the oral administration of 1.8 gm of glucose per kilogram of body weight. The formula is

$$\mathrm{CMI} = \frac{\mathrm{L} - \frac{\mathrm{G}}{10} + 15\,\mathrm{P} - \frac{\mathrm{G}}{10}}{2},$$

where CMI = the index of carbohydrate metabolism, and where G, L, and P, are the levels of glucose, lactic acid and pyruvic acid in mg %, respectively. The possibility of simplifying the formula by using only one of the two metabolites was tempting; i.e., $CMI = L - \frac{G}{10}$ or $CMI = 15\,P - \frac{G}{10}$. Analysis of the data showed that such a simplification was adequate in most cases, and certainly in all cases with a very abnormal index. However, there were many cases where $L - \frac{G}{10}$ did not

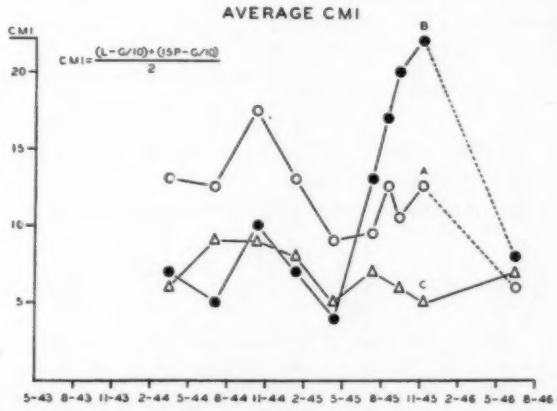

Fig. 3 Indices of carbohydrate metabolism (CMI) for the three dietary groups from March, 1944 to August, 1946. Group A received 400 μg thiamine from May, 1943 to December, 1945. Group B received the same diet plus yeast extract from May, 1943 to June, 1945, at which time their thiamine intake was decreased to 200 μg per day. Group C consumed the regular hospital diet ad libitum. The interrupted lines represent concluding periods of supplementation.

agree with $15\,P - \frac{G}{10}$, due to the fact that a state of equilibrium between lactic and pyruvic acids following exercise had not yet been attained when the blood sample was drawn. In such cases the average of the two gave a more reasonable figure, and one which might be considered more accurate statistically.

The results obtained by calculating the average CMI for groups A, B, and C are shown by graphs in figure 3. Since the glucose-exercise test was not devised until March, 1944, which was 9 months after the start of the dietary phase of the study,

these curves begin at that time. Note that the B group, which was yeast-supplemented until June, 1945, showed a marked increase in CMI when they were placed on 200 μg of thiamine per day, which was half as much as the A group continued to receive. Vitamin supplementation was begun for the indi-

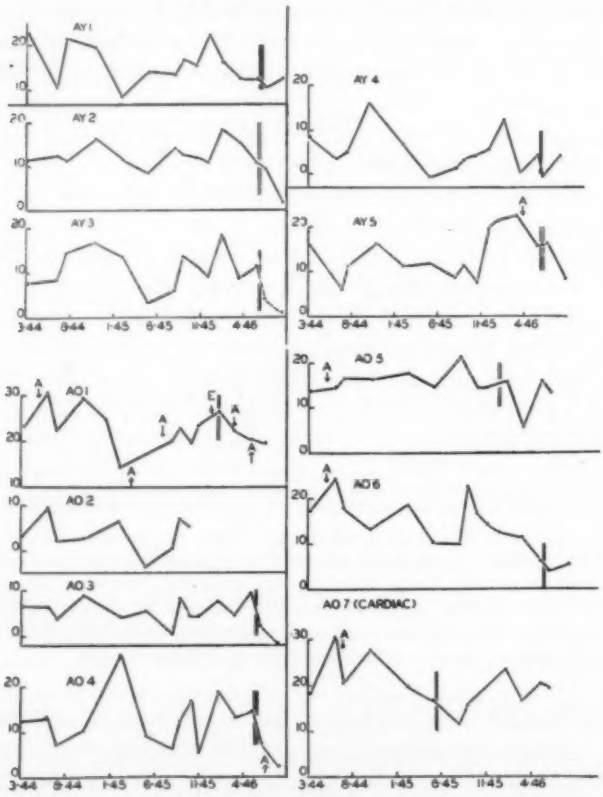

Fig. 4 Indices of carbohydrate metabolism for individual members of the A group. First such test (March, 1944) was not completed until 9 months after the start of the moderately restricted diet (400 μg per day). The multiple vertical lines represent times of supplementation. A and E with descending arrows indicate time at which there was an apparent change in the Achilles tendon reflex and some appearance of edema, respectively. The same symbols with ascending arrows indicate time at which these symptoms began to disappear. Such changes were not marked in this group.

viduals of both these groups after November, 1945. The curve for group C, in which the regular hospital diet was taken ad libitum, remained in a constant narrow range throughout the experiment.

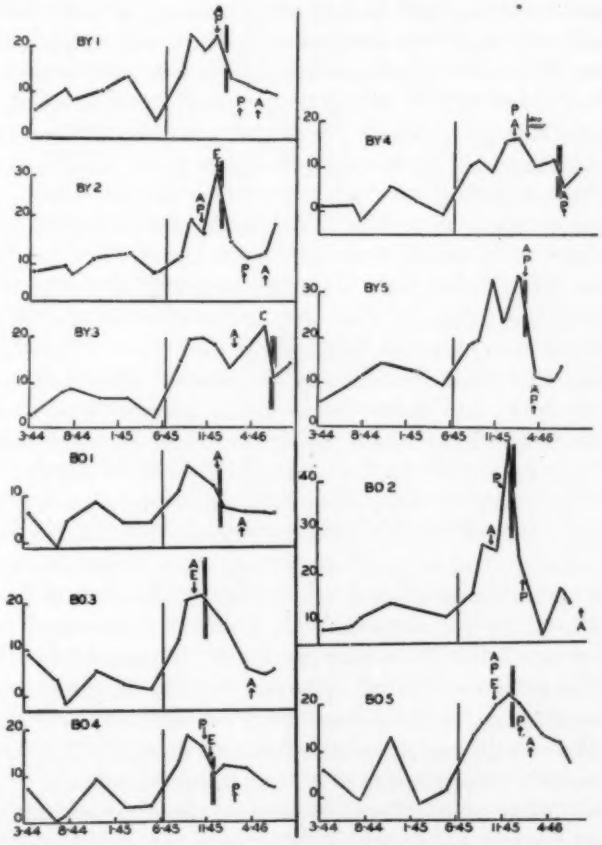

Fig. 5 Indices of carbohydrate metabolism for individual members of the B group. Their depletion period (200 μg per day) started in June, 1945. The multiple vertical lines represent times of re-supplementation. A, P and E with descending arrows indicate first time at which definite signs of clinical deficiency as manifested by Achilles tendon reflex diminution, patellar reflex diminution, and edema, respectively, were noted. Such changes became very marked in this group. The ascending arrows indicate time when these symptoms began to disappear.

The advantage of the CMI over the levels of lactic and pyruvic acids as a measure of thiamine deficiency is demonstrated even better by the results obtained with individual subjects (figs. 4 and 5). There were numerous instances of non-depleted subjects in whom high lactic and pyruvic acid levels were associated with and dependent on simultaneously high levels of glucose. However, in no case did a subject in the B group reach a CMI of more than 15 during the prolonged period of adequate thiamine supply (prior to June, 1945), whereas every subject in this group went higher than 15 within 5 months after severe thiamine depletion and most of them did so within three months. This occurred in every case weeks before clinical symptoms of thiamine deficiency were noted.

The CMI of individuals in the A group (fig. 4) on the borderline thiamine deficiency diet (400 μg per day) fluctuated above and below the value 15, suggesting that these subjects were shifting in and out of a state of biochemical abnormality. The symbols A, P, and E with descending arrows (figs. 4 and 5) indicate the time at which the first clinical signs of thiamine deficiency appeared, as manifested by Achilles tendon reflex diminution, patellar reflex diminution and edema, respectively. The same symbols with ascending arrows indicate the time at which these symptoms disappeared after thiamine was restored by supplementing the diet. The single vertical line represents the time at which the B group was changed from a supplemented diet to the one containing 200 μg of thiamine per day. The multiple vertical lines indicate the time when a daily supplementation with at least 2 mg of thiamine was started. The CMI of all members of the B group promptly fell to below 15 following this renewal of the thiamine supply.

The CMI level of 15 was chosen as the approximate upper limit of normal because this figure was not surpassed by the subjects on an adequate diet and was invariably surpassed by those on a regimen grossly deficient in thiamine. The consistency with which the CMI levels of the B group rose from below 15 when the subjects of this group were placed upon the more depleted diet adds to the significance of the oscillations

observed in the CMI levels of the subjects of the A group on their less depleted diet. As previously mentioned, it is believed that these oscillations signify that the diet of the A group was barely adequate in thiamine and that the subjects of the group were in and out of actual thiamine deficiency. It was observed that the individuals in the A group voluntarily decreased their physical activity periodically during the course of the experiment, this decrease being related to the frequent return of the CMI to a level below 15. Similarly, some of the recovery from the high CMI levels found in the B group at the height of their clinical deficiency and before treatment with thiamine was started, was associated with bed rest. It has been postulated that this demonstrated a decreased need for thiamine when the subjects, as a result of bed rest, were functioning under a minimum "metabolic load."

The CMI of the individuals in the C group are not presented here but it should be mentioned that these subjects, who were on the regular hospital diet, did not reach any values over 15 during the course of the experiment. The average CMI for group C is shown in figure 3.

Test of CMI on population groups

A preliminary test of the procedure based on CMI was made on a group of mental patients in a ward which was known to contain poor eaters. Their CMI was measured before, during and after 8 months of daily supplementation with more than 3 mg of thiamine. These patients, though probably in poor nutritional states, had no incontrovertible signs of thiamine deficiency. Of 33 patients who were available for the 8-month period, 9 started with CMI of 15 or over. After the supplementation period, 7 of these 9 had marked decreases (4 units or more) to below 15, one changed from 30 to 20, and the 9th increased insignificantly from 15 to 16. The CMI of all 9 patients decreased markedly during the first month of supplementation. Of the 24 patients who started at levels below 15, 4 had decreases in CMI of more than 4 units and two had small increases. The remainder were practically unchanged.

A similar study was conducted, simultaneously, on a comparable group of mental patients who worked on the hospital farm and who voluntarily ate more food, probably because of their outdoor activity. These patients showed no significant changes in their CMI. All of these individuals had results below 15 and compared well with the subjects in the C group reported above.

DISCUSSION

To attempt to compare the results obtained in the present study with the reports of other investigators may not be warranted because of differences in the diets, subjects, and duration of the experiments. Nevertheless one might be inclined on the basis of the work now reported to lay greater emphasis on the importance of the results of blood lactic and pyruvic acid levels than would be indicated by the work of Keys, Henschel, Taylor, Mickelsen and Brozek ('45), who claimed that only slight differences were obtained in blood pyruvic acid levels after glucose ingestion. Berryman et al. ('47) reported that the resting and post-exercise pyruvic acid levels were high in some of their experimental subjects.

The fundamental shortcoming of the basal lactic and pyruvic acid levels as a criterion of thiamine deficiency was the large variation in individual figures, regardless of the state of nutrition, as compared with the consistency of figures obtained 60 minutes after glucose ingestion. Although there was a tendency for the basal figures to increase as the deficiency progressed, the significance of this change was not apparent during the early development of deficiency. It was true, however, that many of the resting pyruvate levels were increased after other signs of thiamine deficiency had appeared.

The importance cannot be over-emphasized of evaluating the severity of the "metabolic load" under which a subject is functioning at the time when the blood lactic and pyruvic acids are determined. A level of blood glucose of 200 mg% is a different "load" than a level of 125 mg%. Furthermore, the amount of exercise should be limited. Not only is it simpler

to control the amount of exercise when it is mild, since the factor of volition becomes less important, but avoidance of severe anoxia minimizes the marked variations in levels of lactic and pyruvic acid which are obtained among individuals given strenuous exercise tests. An individual with a less efficient cardiovascular system will have different blood lactic and pyruvic acid levels after severe exercise than a trained athlete after the same exercise, but neither need be thiamine-deficient. Given a smaller "metabolic load" the physically trained and healthy untrained subject tend to give results which approach each other.

Our inclination to ascribe to thiamine the changes in blood lactate and pyruvic values in these experiments is reinforced by other recent observations. A year's subsistence of a group of subjects on a diet containing adequate thiamine but less than 600 μg of riboflavin per day did not produce any significant changes in blood lactic and pyruvic acids, even though characteristic lesions of the skin developed.

Thiamine deficiency is, of course, not the only metabolic situation which will elevate the blood levels of lactic and pyruvic acids. Cardiovascular insufficiencies, liver disorders, infections, thyrotoxicosis, and emotional excitement may all bring about increases. However, all of these conditions can be ruled out by a competent examiner. Data on the effects of disorders of metabolism will be presented in subsequent papers.

SUMMARY AND CONCLUSIONS

1. Thirty-six human subjects receiving diets of known low content of thiamine were studied from the standpoint of the relationship of the levels of lactic and pyruvic acid of the blood to the degree of thiamine deficiency.

2. The normal relationship between blood glucose levels and those of lactic and pyruvic acid was investigated, and the necessity demonstrated for considering this relationship in evaluating levels of blood lactate and pyruvate.

3. In the fasting state and in the absence of exercise there was no consistent rise in the levels of lactic and pyruvic acid

in mild degrees of thiamine deficiency, and it would seem that the organism at rest can maintain the normal equilibrium levels of these metabolites during mild thiamine deprivation. The basal levels were elevated in some cases of more severe thiamine deficiency but, even then, only after pronounced clinical signs of pathology were apparent.

4. The elevation of the lactate and pyruvate values of the blood which followed a combined "metabolic load" composed of ingestion of glucose and mild exercise was found to give more useful data than were obtained either after glucose ingestion alone or after exercise alone.

5. Under the conditions obtaining in the experiments here reported, the blood levels of lactic and pyruvic acid observed under this double "metabolic load" of glucose ingestion and exercise proved to be a means of diagnosing early and mild degrees of thiamine deficiency when these levels were correlated with the glucose level of the blood. The correlation was effected satisfactorily by the formula:

$$\mathrm{CMI} = \frac{\mathrm{L} - \frac{\mathrm{G}}{10} + 15\,\mathrm{P} - \frac{\mathrm{G}}{10}}{2},$$

where CMI stands for the index of carbohydrate metabolism and where G, L and P are levels of glucose, lactic acid, and pyruvic acid, respectively, in milligrams for each 100 ml of blood.

6. The usefulness of the determination for CMI was demonstrated in subjects on controlled thiamine-deficient diets of different severity. The use of the method to pick out thiamine-deficient individuals from population groups was also illustrated.

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GROWTH AND MAINTENANCE OF DOGS FED AMINO ACIDS AS THE SOURCE OF DIETARY NITROGEN

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ONE FIGURE

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Several investigators have studied the replacement of protein in diets of dogs with protein hydrolysates or amino acid mixtures. In 1943 Madden, Carter, Kuttus, Miller and Whipple reported that the 10 "essential" amino acids of Rose ('38) and Rose and Rice ('39) satisfied the requirements of the dog for plasma protein production, weight maintenance, and nitrogen balance. Various partial hydrolysates of protein and a mixture of L- and DL-amino acids have been shown to maintain nitrogen balance when given to dogs by intravenous infusion (Cox and Mueller, '46; Kade, Houston, Krauel and Sahyun, '46; Frost and Risser, '46). Recently, however, it was reported by Miller, Robscheit-Robbins and Whipple ('47) that their doubly depleted dogs "practically always" lost weight when amino acids were supplied in place of protein. Although these authors suggest that an unidentified deficiency may have been responsible for this effect, their food consumption data indicate that the weight loss was associated with a loss of appetite which resulted in an inadequate caloric intake.

The experiments reported in the present paper were designed to determine whether peptide-free amino acid mixtures

satisfy the requirements of the dog for growth and maintenance, and whether exclusion of peptides from the diet results in pathological changes. The amino acid mixtures which were supplied as the sole source of nitrogen were derived from casein and were of the VujN type employed in previous studies (Silber, Seeler and Howe, '46; Smyth, Levy and Sasichak, '47; Eckhardt, Cooper, Faloon and Davidson, '48).

EXPERIMENTAL

Experiment I. Adult dogs

For a period of two weeks, 6 adult mongrel dogs were fed a protein-free basal diet (Silber, Seeler and Howe, '46) plus 1.5 gm of casein per kilogram per day to supply a total of 80 cal. and 210 mg nitrogen per kilogram per day. At the end of this "conditioning" period, two of the dogs were fed amino acid mixture No. 1 (table 1) in place of the casein, two continued to receive casein, and two were supplied neither protein nor amino acids. The daily intake of nitrogen and calories was kept constant throughout the experiment. The amino acid mixture, which was prepared in a 10% aqueous solution, was added to the diet after lyophilization. After the mixture had been supplied in the diet for 40 days, it was given to the same two dogs intravenously (6 mg N per kilogram per minute) for an additional 24 days. Urines and feces were collected continously, and pooled 8-day samples were assayed for total nitrogen and α-amino nitrogen. Blood samples were taken every 8th day for serum protein determinations and for hematological study. Larger samples of blood were taken at the beginning and at the end of the experiment for determination of certain blood constituents (for the methods used see Silber, Porter, Winbury and Clark, '47; for plasma CO_2 capacity see Van Slyke and Cullen, '17). Total fat, nitrogen, and glycogen (Sahyun, '31) contents of the livers were determined on samples taken at autopsy. A number of tissues were fixed in 10% neutral formalin for histological study.

The composition of the amino acid mixture (No. 1) and the average daily intake of each essential amino acid are recorded in table 1. Since preparation of the amino acid mixture involved complete acid hydrolysis of casein, followed by isolation and purification of groups of amino acids, and finally, recom-

TABLE 1

L-Amino acid composition[1] of mixtures and average daily intake of essential amino acids

	ADULT DOGS		PUPS	
	Mixture no. 1	Intake	Mixture no. 2 [2]	Intake
	mg/ml	*mg/kg/day*	*mg/ml*	*mg/kg/day*
Arginine	5.5	78	2.6	173
Histidine	3.2	46	4.1	272
Isoleucine	6.9	98	7.9	525
Leucine	14.2	202	16.4	1,090
Lysine	9.8	139	11.4	760
Methionine	6.0 [3]	121	3.8	252
Phenylalanine	5.9	84	4.0	266
Threonine	2.3	33	1.9	126
Tryptophan	0.75 [4]	11	0.9 [4]	60
Valine	6.3	90	6.8	450
Glycine	22.6		23.6	
Total N	14.9		14.7	
Amino N	12.7		12.1	
Total solids	107.3		103.0	

[1] These preparations contain about 10 mg/ml of non-essential amino acids such as serine, proline, alanine, glutamic acid, and aspartic acid.

[2] Contains about 10% lactic acid.

[3] Contained 2.5 mg of D-methionine in addition.

[4] Added in the form of DL-tryptophan.

bination of the basic fraction and the monoaminomonocarboxylic fraction, the absence of peptides was assured. The mixture gave a negative biuret test and showed no increase in α-amino nitrogen after further acid hydrolysis.

Results

The 4 dogs which received casein or amino acid mixture No. 1 maintained nitrogen balance and body weight. Serum pro-

TABLE 2

Body weights, serum protein concentrations and nitrogen balance in adult dogs

DOG NO.	N SOURCE	INITIAL			40 DAYS			64 DAYS		
		Wt.	Protein	Albumin	Wt.	Protein	Albumin	Wt.	Protein	Albumin
		kg	*gm %*	*gm %*	*kg*	*gm %*	*gm %*	*kg*	*gm %*	*gm %*
721	Casein	7.6	5.2	3.0	8.0	5.7	3.0	8.1	5.4	2.8
798	Casein	7.6	5.2	3.0	7.7	5.5	3.1	7.7	5.6	3.4
753	Amino acids	10.1	5.8	2.5	10.6	5.7	3.2	10.0	5.2	2.9
818	Amino acids	7.2	4.8	2.7	7.6	5.2	3.4	7.4	5.0	2.9
828	None	7.5	5.0	2.8	6.8	4.1	2.0	6.3	4.2	1.9
853	None	13.2	5.3	3.3	10.8	4.2	2.3	9.5	3.8	2.0

Nitrogen intake and excretion, mg per kg initial body weight per day

		CONTROL PERIOD [1]								
		Intake	Urine	Feces	Intake	Urine	Feces	Intake	Urine	Feces
721	Casein	210	219	21	210	192	29	210	172	18
798	Casein	209	208	25	209	156	37	209	124	23
753	Amino acids	216	181	22	215	187	16	215	182	14
818	Amino acids	210	198	27	216	163	23	216	170	18
828	None	208	183	26	5	74	15	5	42	10 [2]
853	None	210	205	20	5	83	20	5	44	10 [2]

[1] Eight-day period.

[2] Lower values here due to decreased food consumption of depleted dogs.

tein concentrations remained essentially unchanged (table 2). Body weight, as well as total serum protein and albumin concentrations, showed a progressive decrease in the protein-depleted dogs.

The fraction of administered nitrogen excreted as the sum of urea, ammonia and amino acid nitrogen was approximately the same whether the amino acids were given orally or intravenously. Hence, nitrogen balance values were independent of the method of administration, although 15 to 20% of the infused amino acids appeared in the urine. When the nitrogen was supplied orally, 75 to 80% was excreted in the form of urea and ammonia; after intravenous infusion, only 63% was excreted in this form. No significant fraction of the amino acid nitrogen administered by either route appeared in the feces, but about 5% of the casein was accounted for as fecal nitrogen (table 2).

During the period of oral feeding, the plasma protein concentrations of both casein- and amino acid-fed dogs increased slightly; after infusion of the amino acids a slight decrease was noted. The plasma albumin concentrations of the dogs fed amino acids in the diet increased, whereas the globulin concentrations decreased (table 2).

The dogs which received amino acids had 11% more hepatic protein, whereas the depleted dogs had 21% less than the casein-fed animals, as indicated by total nitrogen analyses.[1] Furthermore, the ratio of liver weight to body weight was 30% greater in amino acid-fed dogs than in the casein-fed animals.

There were no significant differences between the amino acid- and casein-fed animals in the ratio of plasma cholesterol ester to total cholesterol (0.77), in plasma fibrinogen (265–295 mg%), in serum alkaline phosphatase (1.2–1.8 Bodansky units), in plasma CO_2 capacity (50–54 volumes %), in liver total lipid (9.4–11.5%), in liver glycogen (6.8–7.5%), in plasma

[1] Casein-fed dogs 73 and 74 mg N/100 gm body weight; amino acid-fed dogs 80 and 83 mg N/100 gm; and protein-depleted dogs 52 and 64 mg N/100 gm, respectively.

NPN (22–29 mg%), in plasma glucose (115 mg%), or in plasma creatinine (1.2–1.3 mg%).

Table 3 shows the pertinent hematological data obtained from these dogs. By the 64th day of test, both dogs on the protein-free diet had developed a normocytic anemia, evidence of which had appeared in one of the dogs as early as the 40th

TABLE 3

Hematological data on adult dogs

DIET	DOG NO.	DAY	ERYTHRO-CYTES	HEMO-GLOBIN	HEMATO-CRIT	LEUKO-CYTES PER MM³
			millions/mm³	*gm/100 ml*	vol. %	no.
Casein	721	0	6.3	14.8	49	7,880
		40	6.4	14.0	50	6,800
		64	5.7	14.3	50	8,280
Casein	798	0	6.7	15.0	50	5,040
		40	6.3	15.0	50	7,040
		64	6.2	16.5	53	9,040
Amino acids	753	0	6.4	15.4	52	9,640
		40	6.1	14.0	51	8,040
		64	6.4	14.5	55	12,400
Amino acids	818	0	6.3	15.5	50	8,760
		40	6.9	15.4	52	8,080
		64	6.2	15.0	50	6,920
Protein-free	828	0	6.4	14.5	48	6,320
		40	6.2	13.0	43	6,320
		64	4.8	11.3	38	6,200
Protein-free	853	0	6.8	14.3	49	5,320
		40	6.5	14.5	47	6,920
		64	5.1	11.3	37	7,720

day. The dogs which were given the amino acid mixture, initially in the diet and later by vein, maintained normal ervthrocyte values, as did the casein-fed animals. No significant changes were observed in the total or differential leukocyte counts of any of the dogs.

Gross and microscopic examination of tissues revealed no definite evidence of toxic effects due to the administration of

the amino acid mixture. The liver of one dog on the protein-free diet showed a moderate amount of lipid in the periportal regions. Only a small quantity of hepatic lipid was seen in the other dog on this diet. No lipid was observed in the livers of dogs given casein or amino acids in the diet. The presence of sudanophilic material in Henle's loops and of occasional interstitial round cell collections was noted in the kidneys of dogs of all three groups, and can be ascribed to age and previous history rather than to the specific treatment received during the experiment. An unexplained finding in the kidney of one amino acid-dosed dog consisted of lipid globules present in the glomeruli at the vascular pole. On the average, the number and activity of Malpighian follicles in the spleen, as well as the spleen weight-body ratio, were less in the protein-depleted dogs than in the animals of the other two groups. The amount of tissue hemosiderin varied, but one animal on the protein-free diet showed a rather marked hemosiderosis of liver, spleen and bone marrow. No significant histopathological changes were noted in the heart, lungs, adrenal glands, thyroid glands, pituitary gland, bone marrow, pancreas, urinary bladder, skin, sex organs or gastrointestinal tract of any dog.

Experiment II. Puppies

A litter of 12-week-old Beagle pups was divided into three groups of two each. One group was fed a diet containing 20% of amino acid mixture No. 2 (table 1) ad libitum, the second received an identical amount of diet but with casein (14.7% N) in place of the amino acids, and the third received the protein-free diet only. The 10% solution of amino acids was concentrated under reduced pressure to about 15% of its original volume before being mixed with the diet. After one month, the pups fed the protein-free diet had lost weight and their food consumption was almost negligible. At this time they were supplied the amino acid diet ad libitum.

Every week the pups were weighed and then bled for hematological study. Urines were collected after one month and

again after three months for urinary analyses. At the termination of the experiments, bromsulfalein retention was determined and blood samples were taken for analyses. After receving casein or amino acids for a total of 12 weeks, the dogs were sacrificed and tissues were taken for pathological examination.

Results

The pups given casein or amino acids exhibited comparable rates of growth (fig. 1). The body weights of the protein-depleted pups decreased during the first 4 weeks but showed

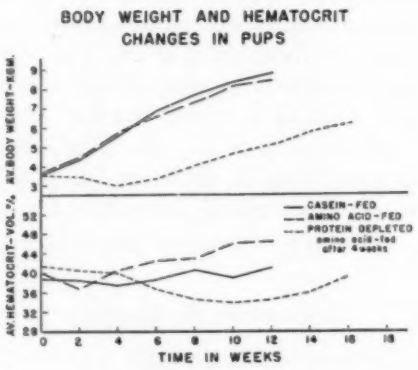
BODY WEIGHT AND HEMATOCRIT
CHANGES IN PUPS
AV. HEMATOCRIT - VOL. %, AV. BODY WEIGHT - KGM.
CASEIN - FED
AMINO ACID - FED
PROTEIN DEPLETED
amino acid - fed
after 4 weeks
TIME IN WEEKS

Figure 1

gradual and consistent gains when the amino acid diet was substituted for the protein-free diet. In 12 weeks the body weights of these dogs increased 100%.

No significant differences between the amino acid-fed and casein-fed pups were found in the ratio of plasma cholesterol ester to total cholesterol (0.76–0.77), in plasma fibrinogen (285–290 mg %), in alkaline serum phosphatase (3.6–3.8 Bodansky units), in plasma NPN (37–38 mg %), in plasma glucose (105 mg %), in plasma creatinine (1.4–1.6 mg %), in serum albumin (3.6–3.8 gm%) or in bromsulfalein retention (10–

15% at 5 minutes, less than 5% after 15 minutes). No significant changes were revealed by the urinary analyses.

At the end of the experiment, the plasma protein concentrations of the casein-fed pups were 5.5 and 5.7 gm %, whereas the amino acid-fed animals had concentrations of 4.8 and 5.1 gm %. However, plasma albumin was increased by amino acid feeding, resulting in albumin/globulin ratios of 2.3 and 2.8 in these dogs in contrast to 1.7 and 2.4 in the casein-fed animals. Analyses of the livers of two of the dogs showed that the total hepatic N was 16.5% greater on a body weight basis after amino acid feeding.

During the 12-week experimental period the casein-fed pups showed an over-all increase in erythrocyte count, hemoglobin concentration and hematocrit values. The pups getting the amino acids, after a slight initial decrease in these blood values, showed an increase which, by the end of the test, was greater than that of the casein-fed controls. A progressive anemia developed in the pups fed the protein-free diet. Even after amino acids were added to their diet, these animals continued to show anemia. However, the beneficial effects of amino acid therapy became evident after the 10th week, and by the 16th week recovery from the anemia was well advanced. The total and differential leukocyte counts in all three groups of animals fluctuated within the normal limits for this species.

Gross and microscopic examinations of tissues from the pups showed, as the only noteworthy change, the presence of moderate amounts of lipid in the periportal regions of the livers of animals which had received the protein-free diet. The ratio of spleen weight to body weight was lowest in this group. The glycogen content of the livers, as shown by Best's carmine stain, was comparable in all animals. No histopathologic changes related to dietary treatment were found in the kidneys, heart, lungs, spleen, thymus, adrenal glands, thyroid glands, pituitary gland, pancreas, urinary bladder, skin, bone marrow, sex organs or gastrointestinal tract of any of the dogs.

DISCUSSION

The growth-promoting properties of amino acids for rats have been the subject of numerous investigations, notably those of Rose ('38). Although similar growth studies in dogs have not been reported, nitrogen balance data are cited by Rose and Rice ('39) to show that the amino acid requirements of the adult dog are qualitatively similar to those of the rat. The experiments of Madden, Carter, Kuttus, Miller and Whipple ('43) support this contention. The feeding of peptide-free amino acid mixtures containing the 10 "essential" amino acids and about 35% of non-essential amino acids has been shown, in the present experiments, to maintain adult dogs and to promote the growth of pups, when supplied with a basal diet also free of peptides.

It is of interest to note that amino acid feeding favored hepatic protein and plasma albumin production in both young and adult dogs. The plasma A/G ratios of the amino acid-fed animals were 25% greater, on the average, than those of the casein controls. Similarly, the hepatic protein was 11 to 16% greater. This difference between the utilization of casein and that of the amino acid mixtures is presumably due to differences in amino acid composition or availability.

Both pups and adult dogs fed a protein-free diet developed anemia. When amino acids were added to the diet of the pups, a delayed but definite increase occurred in erythrocyte, hemoglobin, and hematocrit values. Correction of the anemia was no doubt masked initially by the more rapid correction of the dehydration suffered by these animals as a result of inanition.

In studies of the nutritional value of amino acid mixtures, the adequacy of the basal diet and an optimum caloric intake must be guaranteed. The adequacy of our basal diet for the growth and maintenance of dogs, when casein is included as the dietary protein, has been demonstrated previously in experiments of 2½ to 4½ years' duration (Silber, '44; Seeler and Silber, '45). The daily caloric intake of the adult dogs was

kept constant throughout the experiments, on the basis of 80 cal. per kilogram of initial, fasted, body weight. The caloric intake of the pups ranged from between 225 and 110 cal. per kilogram per day.

When the pups were first offered the amino acid diet, they ate it rapidly and vomitted shortly thereafter, but within several days they learned to consume the diet in small portions and succeeded in retaining sufficient amounts for growth. It is important to note that if the pups had not learned to eat the diet in small portions, it would have been considered inadequate in the usual nutritional sense.

Since both dogs and rats show evidence of a physiological disturbance (nausea or diarrhea) when amino acids or protein hydrolysates are given orally, it seems reasonable to assume that this may be related to limitation of the voluntary intake of amino acid-containing diets and may therefore explain, at least in part, the inability of these animals to grow as rapidly on amino acid diets as on high quality protein diets supplied ad libitum. In general, if an animal fails to consume sufficient diet to satisfy his caloric needs, the diet may appear to be unsatisfactory for growth and nitrogen balance in spite of the presence of all essential factors.

There is no evidence that the physiological disturbance following rapid ingestion of amino acid mixtures is due to unknown toxic factors. Diarrhea has been observed in rats given a variety of protein digests, both enzymatic and acid. It seems likely that the physiological disturbance is due to a nonspecific osmotic effect of the amino acids, and would therefore depend upon the degree rather than the method of hydrolysis.

The data reported in this paper do not exclude the possibility that proteins may contain factors other than amino acids which are beneficial to the animal. Nevertheless, there is no doubt that the growth and maintenance requirements of the dog can be satisfied by feeding a diet containing amino acids as the sole source of nitrogen.

SUMMARY

1. Two adult dogs were fed a peptide-free mixture of amino acids as the sole source of nitrogen, orally for 40 days and intravenously for an additional 24 days. Body weights, serum protein concentrations, and nitrogen balace were maintained. No biochemical, hematological or pathological evidence of toxicity or deficiency was observed.

2. Two pups were fed for 12 weeks a diet containing a peptide-free mixture of amino acids as the sole source of nitrogen. Their average gain in weight was 0.40 kg per week. Pair-fed animals receiving a casein diet gained 0.43 kg per week. There was no indication that the amino acid mixture produced toxic effects, other than an initial transient nausea.

3. Peptides do not appear to be essential for the growth or maintenance of dogs.

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THE INFLUENCE OF COPROPHAGY ON THE BIOTIN AND FOLIC ACID REQUIREMENTS OF THE RAT [1]

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The importance of the intestinal microflora in the nutrition of animals has been demonstrated by many groups of workers. While the animal may benefit to a considerable extent from the activity of the organisms present in the tract, this phenomenon introduces an unknown and complicating factor in the study of the nutritional requirements of the animal. A portion of the metabolic products of the intestinal flora may be absorbed directly from the intestinal tract, and another portion may be obtained by the animal through the ingestion of its fecal matter, a habit known as coprophagy. This phenomenon was first observed by Osborne and Mendel ('11), and it has been studied by many groups of investigators (Steenbock et al., '23; Smith et al., '25; Salmon, '25). Although it was shown by Guerrant, Dutcher and Tomey ('35) that the effects

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of coprophagy were more marked when the carbohydrate of the diet was dextrin, they also recognized some effects due to coprophagy when the diets contained sucrose.

Geyer et al. ('47) recently described the construction of a very satisfactory type of coprophagy-preventing cage. Essentially this cage consists of a horizontal expandable mesh wire tube with provisions for food cup and water bottle on the front end. It allows the rat limited movement without any access to its excreta. In the present paper data are presented on the growth rate and vitamin content of the livers of rats raised in the tube type of cage or in ordinary screen-bottom cages. Some of the diets fed were supplemented with sulfonamide.

EXPERIMENTAL AND RESULTS

Twenty-one-day-old male rats of the Sprague-Dawley strain weighing 40–45 gm were used in all experiments. The rats were housed in individual metal cages (square or tubular as specified). Feed and water were provided ad libitum. The composition of the basal diets is given in table 1. The composition of the water soluble vitamin supplements is listed under the respective headings in table 2. The diets were mixed approximately once weekly and were stored in a refrigerator. In the first experiment comparable groups were housed in square screen-bottom cages and in coprophagy-preventing tube cages, and the effect of supplementing the diets with two different mixtures of water soluble vitamins was studied. Mixture "A" contained thiamine, riboflavin, pyridoxine, calcium pantothenate and choline. Mixture "B" differed from "A" in that it contained in addition the following 5 vitamins: Niacin, folic acid, biotin, *p*-aminobenzoic acid and inositol (table 2). This comparison was carried out with basal diets I and II containing corn oil and butterfat respectively as the sole source of dietary fat (table 1). Thus, there were 8 groups, each containing 12 rats. The results of this experiment are presented in table 3.

It will be noted that confinement in the tube cages reduces the growth of all the rats when compared to that of their mates on the same diets in the square cages. However, attention is directed to the differences between the groups within each type of cage. While the 5 additional vitamins present in supplement "B" improved the growth of the rats on the butterfat and the corn oil diets by 13 and 11% respectively when they were housed in the square cages, the growth increments for the rats in the tube cages were 30 and 29% respectively. There

TABLE 1

Composition of the basal rations[1]

CONSTITUENTS	BASAL RATION NUMBER			
	I	II	III	IV
Casein[2]	20	20	20	20
Salts IV[3]	4	4	4	4
Corn oil[4]	28	..	28	..
Butterfat[5]	..	28	..	28
Sucrose	48	48	..	..
Lactose	..	..	48	48

[1] To each ration were added the following fat soluble vitamins per 100 gm ration: 210 μg 2-Me-1,4-naphthoquinone; 14 μg crystalline irradiated ergosterol; 560 μg β-carotene in the form of a preparation containing 90% β-carotene and 10% α-carotene; and 2.24 mg α-tocopherol.

[2] Extracted for three two-hour periods with boiling alcohol.

[3] Hegsted et al. ('41).

[4] Mazola brand, Corn Products Refining Company.

[5] The butterfat was prepared by melting fresh sweet butter obtained from the University dairy, followed by decantation and filtration.

was no apparent difference in growth due to the type of fat used under the conditions of this experiment.

The second experiment was planned to ascertain which of the 5 additional vitamins was involved in the growth improvement observed in the first experiment. Seven groups of 12 rats each were placed in tube cages and fed basal diet I containing corn oil. Each group received one of the following water soluble vitamin supplements (see table 2): "A," "B" minus folic acid, "B" minus biotin, "B" minus nicotinc acid, "B" minus *p*-aminobenzoic acid, "B" minus inositol and

TABLE 2

Composition of water soluble vitamin mixtures (all values in mg/100 gm ration)

	"A"	"A" PLUS FOLIC ACID	"A" PLUS BIOTIN	"A" PLUS FOLIC ACID PLUS BIOTIN	"B"	"B" MINUS FOLIC ACID	"B" MINUS BIOTIN	"B" MINUS NICOTINIC ACID	"B" MINUS *p*-AMINOBENZOIC ACID	"B" MINUS INOSITOL	LOW
Thiamine	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.10
Riboflavin	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.15
Pyridoxine	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.15
Calcium pantothenate	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	0.75
Choline hydrochloride	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00
Folic acid	...	0.20	...	0.20	0.20	...	0.20	0.20	0.20	0.20	...
Biotin	...	...	0.02	0.02	0.02	0.02	...	0.02	0.02	0.02	...
Nicotinic acid	...	...	...	...	0.60	0.60	0.60	...	0.60	0.60	...
p-Aminobenzoic acid	...	...	...	...	1.50	1.50	1.50	1.50	...	1.50	...
Inositol	...	...	...	...	5.00	5.00	5.00	5.00	5.00	...	...

"B." Seven comparable groups were placed in the square cages. Again all the groups in the square cages grew better than their mates on the same diets in the tube cages. In order to bring out the relative improvement in growth on each supplement in each type of cage, all groups in one type of cage are compared to the negative control, group "A," in the same type of cage, and the values in table 4 represent the increment in per cent growth gain (6-week period) over that of the "A" group. The absolute growth values for the "A" group in the two types of cages are also given.

TABLE 3

Results of experiment 1. (Average[1] gain in gm/6 weeks, 12 rats per group)

Basal ration Dietary fat Water soluble vitamin supplement		I CORN OIL "A"	I CORN OIL "B"	II BUTTERFAT "A"	II BUTTERFAT "B"
Tube cage	gm gain/6 weeks	125 ± 3.87	161 ± 4.73	121 ± 3.38	157 ± 3.83
	growth increment over supplement "A"	...	+ 29%	...	+ 30%
Square cage	gm gain/6 weeks	185 ± 8.8	207 ± 6.9	181 ± 5.05	206 ± 4.93
	growth increment over supplement "A"	...	+ 11%	...	+ 13%

[1] Including the standard error of the mean as calculated from $s_{\bar{x}} = \sqrt{\frac{\Sigma(X - \bar{x})^2}{n(n-1)}}$.

In this experiment the growth stimulation due to the "B" supplement containing all the 5 additional vitamins is again demonstrated to a considerably greater degree in the tube cage (25%) than in the square cage (9%).

In the tube cages, the weight gain of rats on the diets containing supplements "B" minus folic acid and "B" minus biotin were small (9 and 3% respectively) compared to those on the diet containing the "B" supplement. On "B" minus *p*-aminobenzoic acid the gains were intermediate in the tube cages (15% over those of group "A") while in the square

cage they were greater even than on the full "B" supplement. Furthermore, a number of the rats in the tube cages not supplemented with biotin showed the "spectacle eye" symptom.

After finding that growth was suppressed by removing either folic acid or biotin from the "B" supplement, it was of interest to study the effect of these vitamins on growth when they were added to the "A" supplement. In the third experiment 5 comparable groups of 6 rats each were placed in tube cages and fed basal diet I containing corn oil. Each group received one of the following water soluble vitamin

TABLE 4

Results of experiment 2. (Average[1] gains in comparison to gains on supplement "A" in each type of cage, 12 rats per group)

VITAMIN SUPPLEMENT	TUBE	SQUARE
"A" (gm gains)	120.5 ± 5.6	172.0 ± 6.8
	Growth increment as compared to "A"	Growth increment as compared to "A"
"B" minus folic acid	+ 9%	+ 6%
"B" minus biotin	+ 3%	+ 6%
"B" minus niacin	+ 26%	+ 9%
"B" minus p-aminobenzoic acid	+ 15%	+ 13%
"B" minus inositol	+ 22%	+ 9%
"B"	+ 25%	+ 9%

[1] Including the standard error of the mean as calculated from $s_{\bar{x}} = \sqrt{\frac{\Sigma(X - \bar{x})^2}{n(n-1)}}$.

supplements: "A," "A" + folic acid, "A" + biotin, "A" + folic acid + biotin, and "B" (table 2). Similar groups of 6 rats each were placed in the square cages. The results of experiment 3 are given in table 5. The growth of rats in the tube cages was improved 13% by addition of folic acid (supplement "A" + folic acid) and 8% by the addition of biotin (supplement "A" + biotin). When these were added together (suplement "A" + folic acid + biotin) the improvement was 23%, which was comparable to the growth obtained with the addition of the full supplement "B" (22%). In the square cage

the increments were neither as large nor as regular as those observed in the tube cage.

In the 4th experiment studies were carried out to observe the effect on rats in tube cages of corn oil and butterfat diets containing either sucrose or lactose as the source of carbohydrate (diets I, II, III, and IV). The effects of folic acid and biotin were tested with each combination by adding vitamin supplements "A," "A" + folic acid, "A" + biotin, and "A" + folic acid + biotin. Also, supplement "B" containing the 10 B-complex vitamins was added to lactose diets III and IV.

TABLE 5

Results of experiment 3. (Average[1] gains in comparison to gains on supplement "A" in each type of cage, 6 rats per group)

VITAMIN SUPPLEMENT	TUBE	SQUARE
"A" (gm gain)	122 ± 7.3	182 ± 7.8
	Growth increment as compared to "A"	Growth increment as compared to "A"
"A" plus folic acid	+ 13%	+ 8%
"A" plus biotin	+ 8%	+ 4%
"A" plus folic acid and biotin	+ 23%	+ 2%
"B"	+ 22%	+ 3%

[1] Including the standard error of the mean as calculated from $s_{\bar{x}} = \sqrt{\frac{\Sigma(X - \bar{x})^2}{n(n-1)}}$.

Thus a total of 18 groups of 6 rats each were placed in tube cages and fed the above diets. The average gains during the 6-week period are given in table 6.

The results of this experiment confirm those obtained in the previous studies on the effects of folic acid and biotin. On the sucrose diet the differences between the comparable groups were too narrow to allow any statement as to the superiority of one fat over another. On the lactose diets, however, wider differences were observed among all the comparable groups on corn oil and butterfat.

Neilsen and Elvehjem ('42) showed that sulfasuxidine increases the dietary requirement of the rat for biotin and folic acid. In experiment 5 the effect on the vitamin content of the liver of preventing coprophagy was compared with that due to depression of intestinal synthesis by the addition to the diet of 0.5% sulfasuxidine at the expense of sucrose. For comparison, rats were also raised in square cages without sulfonamide. Under each of these conditions butterfat was compared with corn oil. In this experiment low levels of thiamine, ribo-

TABLE 6

Results of experiment 4. (Average[1] gain in gm/6 weeks, 6 rats per group)

BASAL RATION DIETARY CARBOHYDRATES DIETARY FAT	I SUCROSE CORN OIL	II SUCROSE BUTTERFAT	III LACTOSE CORN OIL	IV LACTOSE BUTTERFAT
Supplement				
"A"	116 ± 7.49	119 ± 5.20	59 ± 5.29	76 ± 5.29
"A" + folic acid	129 ± 5.61	138 ± 5.26	75 ± 3.16	82 ± 4.76
"A" + biotin	142 ± 3.94	131 ± 3.77	66 ± 5.09	85 ± 3.81
"A" + folic acid + biotin	144 ± 4.12	136 ± 4.94	81 ± 8.71	90 ± 5.46
"B"	....	....	83 ± 7.46	95 ± 5.68

[1] Including the standard error of the mean as calculated from $s_{\bar{x}} = \sqrt{\frac{\Sigma(X - \bar{x})^2}{n(n-1)}}$.

flavin, pyridoxine, and calcium pantothenate were used (last column of table 2). The experiment was started with 6 groups of 6 rats each, but during its course one rat died in each of the tube cage groups and therefore all the values are based on 5 rats per group. Three rats in the tube cages and 4 in the sulfa-fed groups showed the "spectacle eye" condition. Three rats in the latter groups showed bilateral hair loss on sides and hind quarters. This condition was reported also by Nielsen and Black ('44), who found it could be prevented by the addition to the diet of an inositol supplement.

TABLE 7

Results of experiment 5. (Average [1] gains in gm/6 weeks, and levels of folic acid, biotin and niacin in the livers of the rats, 5 rats per group)

	SQUARE CAGE		TUBE CAGE		SQUARE CAGE AND 0.5% SULFASUXIDINE	
BASAL RATION DIETARY FAT	I corn oil	II butterfat	I corn oil	II butterfat	I corn oil	II butterfat
Growth in gm/6 weeks	92 ± 3.5	114 ± 9.81	53 ± 5.8	60 ± 7.8	68 ± 8.2	59 ± 7.6
μg Folic acid/gm liver	0.43 ± 0.075	0.34 ± 0.068	0.20 ± 0.033	0.21 ± 0.024	0.09 ± 0.016	0.08 ± 0.026
μg Biotin/gm liver	0.82 ± 0.078	0.72 ± 0.081	0.36 ± 0.049	0.38 ± 0.027	0.23 ± 0.026	0.29 ± 0.028
μg Niacin/gm liver	119 ± 7.73	121.5 ± 5.74	120.8 ± 6.03	118.2 ± 5.17	139 ± 5.4	134.6 ± 3.69

[1] Including the standard error of the mean as calculated from $s_{\bar{x}} = \sqrt{\frac{\Sigma(X - \bar{x})^2}{n(n-1)}}$.

At the end of the 6-week growth period the rats were killed by decapitation and the livers removed for vitamin analysis. The method recommended by Olson et al., ('48) was followed for the liberation of the folic acid. Samples of the liver homogenates were incubated at pH 7.0 for 4 to 5 hours, after which the enzymatic reaction was stopped by immersing the tubes in boiling water for three minutes. The folic acid content was determined by using *Streptococcus faecalis* (American Type Culture Collection no. 8043) and the turbidity method of Luckey, Briggs and Elvehjem ('44), in which the medium was modified by the addition of Salts B (Teply and Elvehjem, '45).

The biotin content of the livers was determined by a modification of the method of Wright and Skeggs ('44) in which turbidity was used as the criterion in measuring the growth of *Lactobacillus arabinosus*. For the nicotinic acid assay *L. arabinosus* and the method recommended by Snell ('47) were used.

Growth gains during the 6-week period and the levels of the three vitamins in the livers are given in table 7. Considerably higher levels of biotin and folic acid were found in the livers of rats from the square cages than in those from the tube cages or those fed sulfasuxidine. Of the last two groups, lower levels of these two vitamins were found in the livers of the sulfa-fed animals than in those from the tube cages. This was true on both the corn oil as well as on the butterfat diets. In contrast, the niacin levels of the livers of all groups seem to be similar.

DISCUSSION

It is evident from tables 3, 4, 5 and 6 that under our experimental conditions both folic acid and biotin produce a growth response in rats on diets containing 28% fat and 48% sucrose or lactose. Heretofore the requirement for these two factors by the rat has been clearly demonstrated only when some partially toxic material like a sulfonamide or avidin was incorporated in the deficient diet. While the better growth due to the addition of these two vitamins to the diet is to a certain degree demonstrated in the square cage, their presence in the

diet seems of relatively greater value for the rats deprived of coprophagy. Furthermore, the "spectacle eye" symptom of biotin deficiency (Nielsen and Elvehjem, '41) was observed only in the rats kept in the tube cages.

It would seem that the practice of using a raised screen floor cage, like our square cage, would entirely eliminate coprophagy. However, Geyer et al. ('47) often observed that some rats kept under such conditions obtained feces pellets as they were excreted. Since this habit is more common in some rats than in others, it leads to greater variability in growth data obtained with rats maintained in square cages when the limiting factors are such as can be obtained through coprophagy. Some of the early observers of coprophagy (Steenbock, Sell and Nelson, '23; Guerrant and Dutcher, '32) also noted the variability in the experimental results due to this phenomenon. This is best demonstrated in table 4, where the results of the square cage groups are most irregular.

The growth increment (9%) due to vitamin supplement "B" minus folic acid which contains biotin (table 4) checks well with that (8%) due to vitamin supplement "A" + biotin (table 5). The presence of the three additional vitamins, *p*-aminobenzoic acid, inositol and niacin, in experiment 2 (table 4) seems to exert no apparent effect on growth. However, an inspection of the growth increment (3%) due to "B" minus biotin which contains folic acid (table 4) shows it to be lower than that (13%) due to supplement "A" + folic acid (table 5), which also contains folic acid but no *p*-aminobenzoic acid, inositol or nicotinic acid. This might be due to the variations regularly encountered in such biological experiments. On the other hand, a possible explanation is that the additional vitamins (*p*-aminobenzoic acid, inositol and nicotinic acid) may accentuate the growth of microorganisms which tend to be folic acid users and thus deprive the animal of the folic acid obtained in the tube cage by absorption from the intestinal tract.

The liver biotin and folic acid values represented in table 7 should not be considered a quantitative estimation of the intestinal synthesis of these two vitamins, their absorption di-

rectly from the ceca, or the amount obtained by the rat through his habit of coprophagy, because (a) there is some synthesis in the gastrointestinal tract in spite of the sulfa drug, (b) part of the vitamins synthesized by some microorganisms are destroyed by others whether sulfa is present or not, and (c) the quantitative aspects of these phenomena differ too greatly among individual rats to allow us to make conclusive statement on the basis of the small number of rats used in this experiment. However, the values found are very interesting and significant in demonstrating these phenomena at least qualitatively under the three conditions: Square cage, tube cage and square cage with sulfasuxidine.

Salmon ('47) found that high levels of fat tended to counteract a deficiency of niacin. He believes that this is a nicotinic acid-sparing effect of fat. Hankes et al. ('48) reported similar observations. In addition to the possibility of the sparing effect, they suggest also the possibility of better synthesis or decreased destruction of niacin by the intestinal flora when a high fat ration is fed.

Krehl et al. ('45) showed that tryptophan can replace nicotinic acid in counteracting the growth retardation caused by corn grits in a low protein ration. Rosen et al. ('46) concluded from their excretion studies that tryptophan may be the important precursor of nicotinic acid synthesis in the rat. Similar observations were made also by Singal and his group ('46). Our data on the liver content of nicotinic acid seem to be in line with these studies. The niacin values shown in table 7 indicate that neither the type of dietary fat (28% level) nor the abiltiy or inability of the rat to consume its feces affected the amount of liver niacin, under our conditions. Rather, it seems to have been determined by the 20% casein the rats received in their diets, which also contained 28% of fat.

SUMMARY

1. Rats fed purified diets and kept in coprophagy-preventing tube cages grew significantly better when folic acid and biotin were added to the diet than when these vitamins

were omitted. Inositol, *p*-aminobenzoic acid, and niacin did not affect the growth rate of these rats. Biotin and folic acid produced only slight and irregular improvement in the growth of rats kept in ordinary screen-bottom cages.

2. The separate effects of coprophagy-prevention and the addition of sulfasuxidine were reflected also in the biotin and folic acid content of the liver, but not in the nicotinic acid content.

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ORAL MANIFESTATIONS IN RATS FED SYNTHETIC DIETS DEFICIENT IN PANTOTHENIC ACID AND BIOTIN [1]

I. METHODS AND GENERAL GROSS SYMPTOMS

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TWO FIGURES

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The purpose of this study was to observe the oral lesions produced by a synthetic diet in which calcium pantothenate, panthenol, biotin and folic acid were the experimental variables. The replacement effects of these agents were noted by their use singly and in various combinations. Skin manifestations and weights were also considered.

Ziskin, Stein, Gross and Runne ('44, '45, '47) reported the production of mouth ulcers, hyperkeratinization of the oral mucous membrane, decolorization of the incisors, and marked gingival and periodontal necrosis in rats by the addition of zinc carbonate to a diet which was suboptimum in pantothenic acid. Similar results were obtained by them in rats fed a synthetic diet totally deficient in pantothenic acid but ade-

[1] This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service, and in part by a grant from the Roche Anniversary Foundation. Presented at a meeting of the New York Section of the International Association for Dental Research, October 30, 1947, and at the general meeting of the International Association for Dental Research, June 18–20, 1948. Calcium pantothenate, panthenol, biotin and the other vitamins used in this study were provided by Hoffmann-LaRoche Inc., Nutley, New Jersey.

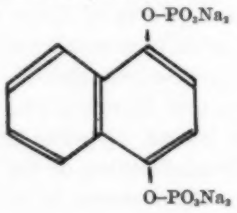
quate in every other respect, although not as many animals were affected. Wainwright and Nelson ('45) reported generally comparable results in rats on a pantothenic acid deficient diet without the addition of zinc carbonate.

TABLE 1

Composition of basal diets

CONSTITUENTS	BASAL DIET		VITAMIN SUPPLEMENTS ADDED TO COMPLETE BASAL DIETS	AMOUNT PER ANIMAL PER DAY
	1	2		
	%	%		
Casein, vitamin-free	24		Thiamine hydrochloride	20 μg
Sucrose	64		Pyridoxine hydrochloride	20 μg
Egg white		30	Riboflavin	40 μg
Dextrin		55	Nicotinic acid (niacin)	1 mg
Crisco	5	8	Choline chloride	4 mg
Cod liver oil	3	3	Para-aminobenzoic acid	250 μg
Salt mixture	4	4	i-Inositol	500 μg
			Vitamin E [1]	400 μg
			Vitamin K [2]	80 μg

[1] d-l-Tocopherol, phosphoric acid disodium; synthetic water soluble vitamin E.
[2] Synkayvite — synthetic vitamin K (water soluble).



EXPERIMENTAL

Twenty groups of rats, 18 of 10 animals each and two of 16 animals each, were used in this study. There were equal numbers of males and females in each group. The weanling rats were maintained in litters with the mother until they attained the desired weight, which was 40 ± 2 gm, at which time they were weaned and placed on the synthetic diets.

The rats were housed in individual cages with wire-mesh bottoms. The wire-mesh reduced the practice of coprophagy,

but did not entirely prevent it. Water and the respective basal diets as shown in table 1 were supplied in unlimited quantities.

The preparation of the vitamin supplements (table 1) which completed the basal diets, and the manner of feeding these

TABLE 2

Combinations of experimental variables added to basal diets

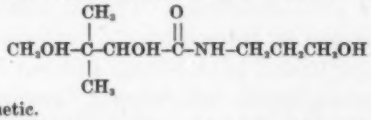
DIETS	GROUP [1]	BIOTIN,[2] 2 μG DAILY	CALCIUM PANTOTHENATE,[3] 150 μG DAILY	PANTHENOL,[4] 150 μG DAILY	FOLIC ACID,[5] 2 μG DAILY	DESOXYCORTICOSTERONE-ACETATE [6]
	1	+	+	0	+	0
	2	+	0	+	+	0
	3	0	0	0	0	0
	4	+	0	0	0	0
	5	+	+	0	0	0
Basal	6	+	0	+	0	0
diet	7	+	0	0	+	0
1	8	0	0	0	0	—[6]
	9	0	0	0	+	0
	10	0	+	0	0	0
	11	0	0	+	0	0
	12	0	+	0	+	0
	13	0	0	+	+	0
	14	0	0	0	0	0
	15	+	0	0	0	0
Basal	16	0	+	0	0	0
diet	17	0	0	+	0	0
2	18	+	+	0	0	0
	19	+	0	+	0	0
	20	0	0	0	+	0

[1] Each group consisted of 10 animals except groups 3 and 14, which had 16 animals each.

[2] d,l-Biotin, synthetic.

[3] d,l-Calcium pantothenate (crystalline).

[4] d-Panthenol, synthetic (pantothenyl alcohol = alcohol of pantothenic acid).



[5] Folic acid, synthetic.

[6] Synthetic desoxycorticosterone-acetate. In group 8, 4 animals received 0.1 mg, three animals received 0.2 mg, and three animals received 0.3 mg daily.

supplements were as follows: A stock solution was made by dissolving 116.5 mg thiamine hydrochloride, 116.5 mg pyridoxine hydrochloride, 233 mg riboflavin, 5.825 gm nicotinic acid, 23.3 gm choline chloride, 1.456 gm para-aminobenzoic acid, 2.912 gm *i*-inositol, 2.33 gm vitamin E, and 466 mg vitamin K in distilled water at about 40°C. and diluting to 500 ml. One part of this solution was diluted with 4 parts of a 10% sucrose solution and 1 ml of the resultant preparation fed in supplement cups to each rat three times a week. This supplied the amounts indicated in table 1. A similar procedure was followed for the experimental variables listed in table 2. The fat soluble vitamins A and D were provided in the cod liver oil incorporated in the basal diets. Diets were made up in sufficient quantity for a week's feeding. The experimental variables that were added to the two basal diets each week for the 20 groups of rats are shown in table 2.

By the end of the 12th week of the experiment the rats either died or were sacrificed. The tongue, teeth and supporting structures, and viscera were prepared for histological investigation. The results of this part of the study will be reported later.

RESULTS AND DISCUSSION

Gross symptoms

During the course of the experiment the animals were examined for the symptoms which have been previously noted in the rat in pantothenic acid and biotin deficiency. These are listed in table 3. An evaluation of these symptoms based on severity, time of appearance and duration is given in this table.

The rusting of the fur appeared to be a symptom associated with pantothenic acid deficiency. This is shown by the fact that it was prevented by the addition of calcium pantothenate to the diet (groups 10 and 16) and was present in those groups where calcium pantothenate was absent (groups 3, 4, 7, 8, 9). However, the addition of biotin to basal diet 2 seemed to increase the need for pantothenic acid in this regard; i.e., when

TABLE 3

Gross symptoms observed on various diets[1]

TABLE 3

Gross symptoms observed on various diets [1,2]

	BASAL DIET 1 PLUS EXPERIMENTAL VARIABLES AS INDICATED													BASAL DIET 2 PLUS EXPERIMENTAL VARIABLES AS INDICATED						
	Control: Biotin, Ca pantothenate and folic acid	Control: Biotin, panthenol and folic acid	No experimental variables	Biotin	Ca pantothenate and biotin	Biotin and panthenol	Biotin and folic acid	Desoxycorticosterone	Folic acid	Ca pantothenate	Panthenol	Ca pantothenate and folic acid	Panthenol and folic acid	No experimental variables	Biotin	Ca pantothenate	Panthenol	Biotin plus Ca pantothenate	Biotin plus panthenol	Folic acid
Group number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Arched back [3]																1		1		5
Dermatitis [3,4]	1		2	3							1		1	2		2	1	1		
Cropped fur		±	+++	+	±	±	++	+++	+++	±	±	+	+	+++	+	++	++	+	±	+
Rusting fur		+	+++	+	±	±	++	++	++	±	+	+	+	±	+	±	±	+	±	±
Alopecia body	±		++	++	±	±	+	++	++	±	±	±	±	++	±	++	++	+	±	++
Alopecia eyes			±	±										±						++
Alopecia mouth			+				±		±					+	±	++	++	+	±	+
"Spectacle-eyes"			±	±			±	±	±					+	±	++	+	±		±
Caked whiskers			±				±	±	±					+	±	++	++	±		±
Crusting of ears and toes			±	±		±	±	±	±	±	±	±	±	++	±	++	++	±	±	++
Scaling of paws			+	+			+	++	++			±	±	+	±	+	+	±	±	+
Crusting of tail			+	±		±	±	±	±	±	±	±	±	+++	+	+	+	+	±	+++
Decolorization of teeth			±	±		±		±	±	±	±	±	±		±		±	±	±	
Porphyrin nose			+	+			+	+	+			±		±	±	+	+	±		±
Fissures at angles of mouth			++	±			+	+	+	±	±		±	++		+	+	+		+
Deposit on teeth			±	±			±	+	±	±				±	±		±			
Edema of mouth														+						±
Swollen eyelids														+		±	±			

[1] The symptoms noted in this table are not due to an amino acid deficiency since the proteins in both diets are regarded as "complete proteins" at the levels used.

[2] Blank space indicates absence of the specific symptoms. ± = Present but at negligible levels. + = Mild. ++ = Moderate. +++ = Severe.

[3] Numbers refer to number of animals in each group with the respective condition.

[4] Dermatitis consisted of a crusting type of lesion.

neither biotin nor calcium pantothenate was added to basal diet 2 (group 14) rusting did not appear. It did appear when biotin alone (group 15) was added or when biotin and calcium pantothenate (group 18) were added. The "spectacle-eye" condition, caked whiskers, crusting of the ears and tail, edema of the mouth and swollen eye-lids were associated chiefly with the biotin deficient diet, basal diet 2. Porphyrin around the nose was more marked on basal diet 1, while alopecia, scaling of paws and fissures at the angles of the mouth, appeared with equal frequency on both diets.

When calcium pantothenate was added to basal diet 1 (group 10) most, but not all, of the symptoms were alleviated. The addition of panthenol (group 11) produced about the same effects as the addition of calcium pantothenate.

The addition of biotin to basal diet 1 (group 4) led to improvement as regards cropping of the fur, rusting of the fur, crusting of the tail and fissures at the angles of the mouth.

The addition of calcium pantothenate or panthenol, and biotin and folic acid to basal diet 1 (groups 1 and 2) gave the best results. The addition of biotin and folic acid (group 7) was not as effective as biotin alone (group 4). The addition of folic acid (group 9) to basal diet 1 provided no relief.

The animals receiving calcium pantothenate and folic acid (group 12) and the animals receiving panthenol and folic acid (group 13) were not protected as well as those animals receiving calcium pantothenate or panthenol alone (groups 10 and 11).

The addition of biotin to basal diet 2 (group 15) materially alleviated the symptoms but did not offer complete protection. The addition of calcium pantothenate (group 16) or panthenol (group 17) to basal diet 2 was not found to be beneficial. The effect of biotin and calcium pantothenate combined (group 18) did not vary greatly from that seen when biotin alone was added, although the combination of biotin and panthenol (group 19) appeared to be somewhat more effective. Folic acid was not found to be an effective supplement in biotin deficiency.

Sex differences were noted on several diets. The males were more severely affected than the females in group 3, basal diet 1, no supplements; group 7, basal diet 1, with biotin and folic acid added; group 14, basal diet 2, with biotin added; and group 18, basal diet 2, with biotin and calcium pantothenate added. The differences between the sexes, however, were not marked.

Autopsy findings

The mouth symptoms observed at autopsy in groups fed basal diet 2 consisted chiefly of bullous lesions on the tongue. Both clear and hemorrhagic bullae were seen. In some animals a pattern somewhat resembling "geographic tongue" in man was found. The groups affected most severely with tongue symptoms were as follows: group 7, on basal diet 1, with biotin and folic acid added (necrotic lesions); group 14, on basal diet 2, with no experimental variables (bullous lesions); group 16, on basal diet 2, with pantothenic acid added (bullous lesions); and group 20, on basal diet 2, with folic acid added (bullous lesions). A few isolated tongue lesions were found in groups 8 (increased redness), 12 (ulcers) and 17, 18, 19 (blisters).

Weight

Figures 1 and 2 show the weight curves based on the average weights of the animals in each group taken at weekly intervals ($\pm$ 3 days) following the first 4 weeks of the experiments.

Basal diet 1 (group 3) unsupplemented by the dietary variables of interest produced a severe depression in weight which was slightly improved in group 4 (biotin added), while the growth curve was somewhat depressed in the groups that received folic acid (groups 7 and 9) and desoxycorticosterone-acetate (group 8). Both calcium pantothenate (group 10) and panthenol (group 11) proved to be completely satisfactory supplements in this regard.

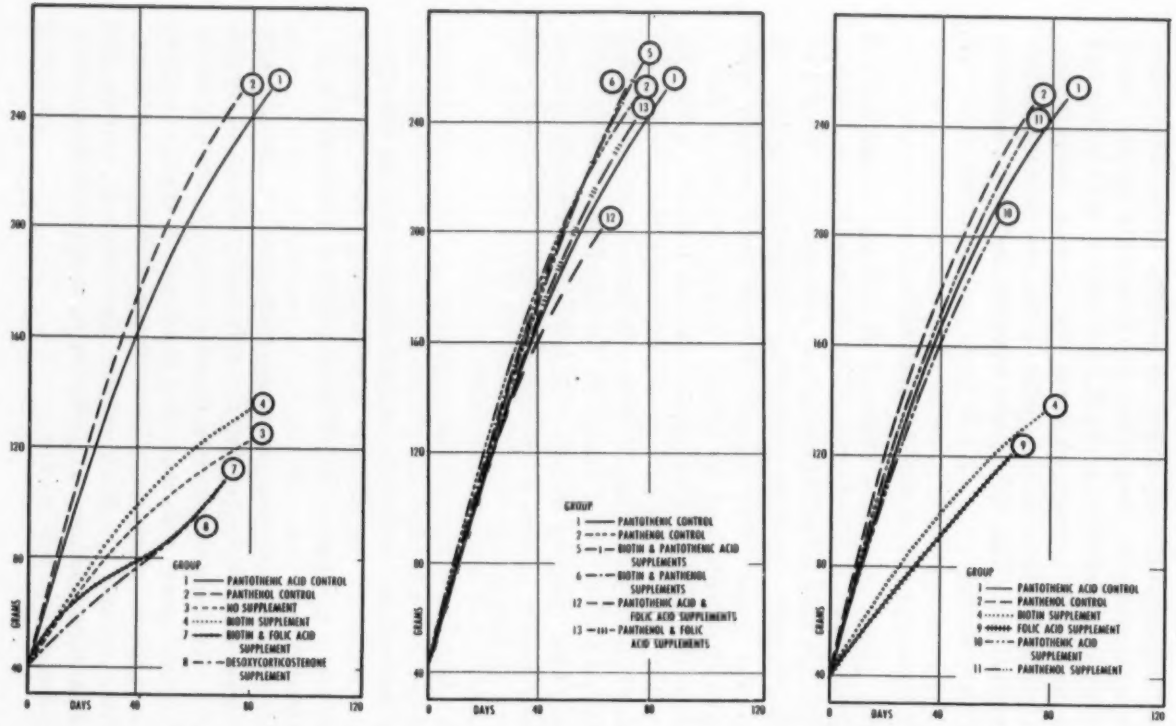


Fig. 1 Growth curves of groups of rats fed diets with or without supplements indicated.

In the biotin deficient animals fed basal diet 2 the addition of biotin (group 15) appeared to provide complete protection, the addition of calcium pantothenate (group 16) or panthenol (group 17) provided slight protection, and again folic acid

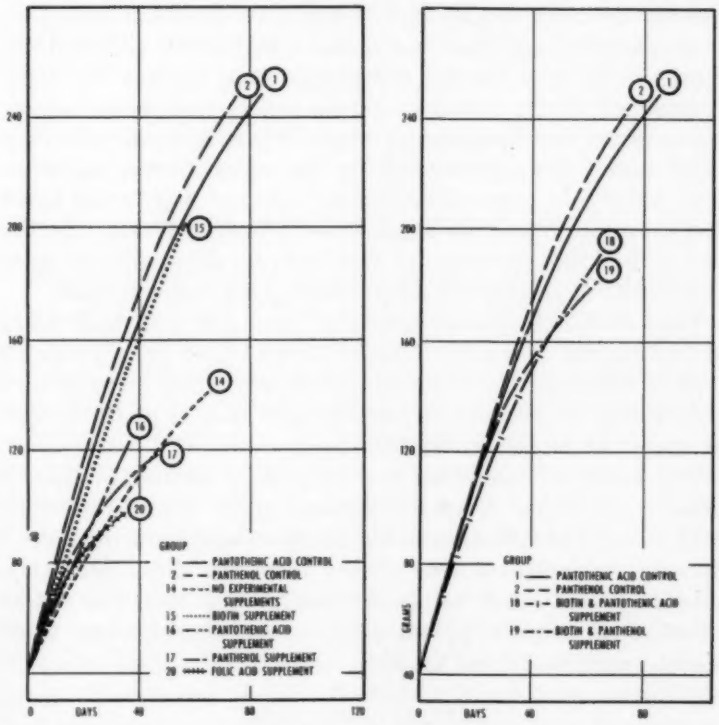


Fig. 2 Growth curves of groups of rats fed diets with or without supplements indicated.

(group 20) depressed the weight curve. The combinations of biotin and calcium pantothenate (group 18) and biotin and panthenol (group 19) were found to have no more effect than biotin alone. The findings as to weight did not exactly parallel those relating to the skin or oral symptoms.

SUMMARY

This investigation is concerned with the changes produced in rats fed one of two synthetic diets complete in every respect except for pantothenic acid, panthenol (a synthetic alcohol of pantothenic acid), biotin and folic acid, which were the experimental variables. Basal diet 2 differed from basal diet 1 in that egg white replaced the casein and dextrin replaced sucrose. With each diet the 4 vitamins were omitted for some groups of animals, added singly for others, and given in combinations to the remaining groups. Those animals receiving basal diet 1 unsupplemented by the experimental variables were chiefly on a pantothenic acid deficient diet, while basal diet 2, unsupplemented, emphasized a lack of biotin. By the end of the 12th experimental week the rats either died or were sacrificed. Skin manifestations and weights were studied.

Unprotected groups subsisting on basal diet 1 showed ulcers of the tongue in various sizes characterized by a granular type of necrosis. Unprotected groups subsisting on basal diet 2 showed grossly clear and hemorrhagic vesicles on the tongue of subepithelial or subdermal origin.

The action of panthenol was in general similar to that of calcium pantothenate under all conditions. Biotin plus folic acid was not as effective as biotin alone when calcium pantothenate or panthenol was absent from the diet. Similarly, calcium pantothenate or panthenol plus folic acid was not as effective as calcium pantothenate or panthenol alone when biotin was absent from the diet.

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LONG-TERM EXPERIMENTS AT OR NEAR THE OPTIMUM LEVEL OF INTAKE OF VITAMIN A [1]

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General acceptance of the principle that with some nutrients there are important differences between the amounts needed for merely or barely adequate, as compared with optimum nutrition, naturally leads to a growing interest in a related concept. When is there simply a *point*, and when is there a *plateau*, of optimum response? And when the latter is the case, is the plateau equally wide with respect to different criteria of nutritional well-being? The data which follow are offered as throwing some light upon these questions as they apply to vitamin A.

Previous work in this Department has shown that when rats of like age, sex, size and genetic and nutritional background are given diets alike in other respects but containing, respectively, three, 6 and 12 I.U. of vitamin A per gram of air-dry food mixture, the increases of intake of this vitamin resulted in increased gains of body weight with decreased coefficients of variation (Sherman and Campbell, '45), as well as larger bodily stores of the vitamin and increased length of life (Caldwell, MacLeod and Sherman, '45; Campbell, Udiljak, Yarmolinsky and Sherman, '45; Sherman, Campbell, Udiljak and Yarmolinsky, '45).

[1] Aided by grants from the Nutrition Foundation, Inc., to Columbia University.

The present paper reports the results of comparisons of the same and other responses to feedings at the two levels of 12 and 24 I.U. of vitamin A per gram of air-dry food, or approximately 3.2 and 6.4 I.U. per Calorie, respectively. As judged by several criteria of nutritional well-being throughout the life cycle, response as a whole is more favorable at 12 I.U. than at either the 6 or the 24 I.U. level of intake of vitamin A. Hence the findings here reported compare the effects of an approximately optimum intake of vitamin A with those of a two-fold higher level of intake.

EXPERIMENTAL

The general methods used were those employed in the other feeding researches published from this Department at intervals since 1921, and the experimental animals were rats from the same long-established, laboratory-bred Osborne-Mendel branch of the Wistar Institute strain. The basal diet was our Laboratory Diet No. 16, often, for convenience, referred to as diet A. It consists of five-sixths ground whole wheat and one-sixth dried whole milk, with table salt 2% of the weight of the wheat, and pure water ad libitum. The extra vitamin A was added in the form of high grade cod liver oil, of such potency that the amount of food mixture it displaced was negligible, averaging only about one part in 400. As previous experiments have consistently shown that animals bred, housed, handled and fed as were those here used do not respond to added vitamin D, this was deemed adequate evidence that their supplies of vitamin D are such that the benefit resulting from added cod liver oil is attributable to its vitamin A. While obviously it is conceivable that some unknown constituent still present in highly refined cod liver oil may be a significant factor contributing to these results, there is no positive indication of any such substance. Diet 361 had 12 I.U. and diet 362 had 24 I.U. of vitamin A per gram of air-dry food. Both averaged 14.2 to 14.4% of protein.

The rats were weighed weekly throughout their lives, and the pregnant females were weighed every second day.

The air-dry food mixture was always available to the animals and records of their food consumption were kept from the age of 28 days throughout the lives of the first generation and from the 28th day to the 56th day of life in the second.

Of diets 361 and 362 containing respectively 12 and 24 I.U. per gram of air-dry food, the rats ate essentially the same amounts: 0.49 to 0.42 Cal. per gram of body weight per day at 5 to 7 weeks of age; 0.35 to 0.25 at two to three months; 0.23 to 0.21 at 4 to 6 months; and about 0.20 at 6 months and over. Early growth was very slightly more efficient, both per 1,000 Cal. and per gram of protein consumed, on the diet having 12 than on that having 24 I.U. per gram.

The experimental animals were divided into matched lots so that those receiving one of the two diets which were being compared were exactly duplicated by those receiving the other diet. Each experimental lot consisted of three females and two males. As each female showed pregnancy she was separated from the group and kept in a cage by herself until her young were 4 weeks old; then she was returned to her original cage. The offspring were used in part to make up second generation breeding lots and in part for the studies of body storage reported below.

Growth, breeding records, and length of life

The data on growth, breeding records, and length of life yielded by strictly comparable lots of animals fed, respectively, diet 361 with 12 I.U. and diet 362 with 24 I.U. of vitamin A per gram are summarized in table 1.

Storage of vitamin A in the liver

Having previously studied bodily storage at ages up to 300 days (Campbell, Udiljak, Yarmolinsky and Sherman, '45), the experiments here reported show the amounts of vitamin A found in the livers of rats of both sexes at the ages of 500 and of 700 days. As in the work of the above authors, the determinations were made by the single feeding method described

TABLE 1

Growth, weight, breeding records, and length of life of rats on two diets differing in vitamin A content

	ON DIET 361 WITH 12 I.U./GM			ON DIET 362 24 I.U./GM		
	No. of cases	Mean with its P.E. [1]	C.V. [2]	No. of cases	Mean with its P.E. [1]	C.V. [2]
Gain in wt., 28th to 56th day of life						
Males (gm)	24	65.6 ± 0.71	8	24	65.9 ± 1.02	11
Females (gm)	36	54.3 ± 0.62	10	36	54.6 ± 0.56	9
Wt. at 84 days of age						
Males (gm)	24	184.0 ± 1.95	8	24	184.7 ± 1.68	7
Females (gm)	26	151.3 ± 1.40	7	29	152.1 ± 1.30	7
Wt. at 100 days						
Males (gm)	24	217.3 ± 1.93	7	24	213.3 ± 2.25	8
Females (gm)	25	167.3 ± 1.55	7	28	170.8 ± 1.69	8
Wt. at 120 days						
Males (gm)	24	248.3 ± 2.00	6	24	240.3 ± 2.86	9
Females (gm)	33	179.8 ± 1.63	8	33	180.3 ± 1.79	9
Wt. at 150 days						
Males (gm)	24	277.5 ± 2.17	6	24	269.7 ± 3.03	8
Females (gm)	25	186.6 ± 1.61	6	32	190.6 ± 1.49	7
Wt. at 180 days						
Males (gm)	24	298.5 ± 2.63	6	24	290.6 ± 3.05	8
Females (gm)	31	198.5 ± 1.86	8	33	201.0 ± 2.13	9
Wt. at 210 days						
Males (gm)	24	313.5 ± 3.21	8	24	302.0 ± 2.81	7
Females (gm)	27	205.2 ± 1.85	7	32	208.9 ± 1.99	8
Wt. at 240 days						
Males (gm)	24	323.0 ± 3.87	9	23	309.6 ± 2.89	7
Females (gm)	33	205.8 ± 1.65	7	32	211.1 ± 2.04	8
Wt. at 300 days						
Males (gm)	24	329.4 ± 4.08	9	22	320.5 ± 3.16	7
Females (gm)	30	218.4 ± 1.95	7	32	216.2 ± 1.81	7
Wt. at 365 days						
Males (gm)	24	333.5 ± 4.21	9	22	318.5 ± 3.21	7
Females (gm)	27	220.7 ± 2.62	9	35	220.6 ± 1.93	8

TABLE 1 *(continued)*

	ON DIET 361 WITH 12 I.U./GM			ON DIET 362 24 I.U./GM		
	No. of cases	Mean with its P.E. [1]	C.V. [2]	No. of cases	Mean with its P.E. [1]	C.V. [2]
Wt. at 500 days						
Males (gm)	24	311.0 ± 6.57	16	19	305.7 ± 4.78	10
Females (gm)	29	234.1 ± 2.35	8	31	231.9 ± 2.74	10
Wt. at 600 days						
Males (gm)	17	310.6 ± 8.29	16	15	287.3 ± 5.85	12
Females (gm)	30	236.0 ± 2.28	8	27	228.3 ± 2.73	9
Wt. at 700 days						
Males (gm)	14	291.0 ± 8.49	16	8	268.0 ± 10.95	17
Females (gm)	26	225.6 ± 2.63	9	25	217.0 ± 3.42	12
Age at birth of first young (days)	36	112.2 ± 2.84	23	35	112.6 ± 3.25	26
Number of young borne per female	36	42.3 ± 1.63	35	36	32.3 ± 2.04	57
Number reared	36	31.6 ± 1.73	49	36	24.4 ± 1.80	66
Reproductive life of females (days)	36	386.9 ± 15.16	35	36	292.3 ± 19.09	59
Length of life						
Males (days)	24	715.9 ± 14.93	15	24	646.7 ± 20.54	23
Females (days)	36	794.1 ± 18.01	20	36	777.0 ± 18.27	21

[1] P.E. = the "classic" probable error of the mean.
[2] C. V. = coefficient of variation.

by Sherman and Todhunter ('34). The sexes were kept separate in these studies. The results are shown in table 2.

DISCUSSION

In the experiments here reported the rates of early growth and their variability were consistent with those found by Sherman and Campbell ('45). With the males, but not the females, the highest intake level was associated with a very slightly less rapid early growth.

In later growth the females showed practically the same results on the two intake levels here studied, while the males grew very slightly less on the higher level. Coefficients of variation were low for both levels and both sexes.

Increasing the vitamin A content of the diet from 12 to 24 I.U. per gram appears to have shortened the length of life. A much larger proportion of males died under the age of two years on the intake level of 24 I.U. than on that of 12 I.U.,

TABLE 2

Vitamin A in I.U. per gram of rat liver (fresh weight)

	MALES			FEMALES		
	No. of cases	Mean with its P.E. [1]	C.V. [2]	No. of cases	Mean with its P.E. [1]	C.V. [2]
At 500 days on:						
Diet 16 (3 I.U.)	9	6 ± 1.3	101	5	2 ± 0.6	105
Diet 360 (6 I.U.)	10	71 ± 6.4	43	16	165 ± 6.0	22
Diet 361 (12 I.U.)	19	771 ± 24.6	21	21	827 ± 40.6	34
Diet 362 (24 I. U.)	10	1453 ± 80.9	27	10	1485 ± 49.4	16
At 700 days on:						
Diet 361 (12 I.U.)	6	1333 ± 141.3	39	11	1373 ± 93.9	34
Diet 362 (24 I.U.)	3	2700 ± 88.6	9	9	3106 ± 122.5	18

[1] P.E. = the "classic" probable error of the mean.
[2] C.V. = coefficient of variation.

which appears to be optimum. With the females this difference was not as great. Females on the two levels bore their first young at the same age, but the reproduction record as a whole was less variable and averaged distinctly better on the (presumably optimum) level of 12 I.U. than on the two-fold higher level of 24 I.U. per gram of dry food.

While based upon differently determined criteria of minimum adequacy, our findings as to the vitamin A requirements

(minimum and optimum) of the rat are in general agreement with those of Goss and Guilbert ('39), of Lewis, Bodansky, Falk and McGuire ('41) and of Paul and Paul ('46). The highest concentrations of vitamin A in fresh liver tissue found for rats in this study are about one-fifth those found for bears and seals by Rodahl and Moore ('43).

On the diet containing 3 I.U. of vitamin A per gram of air-dry food, body stores were negligible. On intake levels of 6, 12, and 24 I.U. per gram of food, the body stores of vitamin A increased progressively with each increase of the intake level. Experiments at the 12 and 24 I.U. levels showed that body stores were higher at 700 days than at 500 days of age. These results were true of both sexes.

SUMMARY

In experiments with rats, an increase of vitamin A in the diet from 12 I.U. to 24 I.U. per gram did not measurably influence growth between the ages of 28 and 56 days. This was also true of later growth and adult size in the females. Males grew to slightly lower adult weights on the diet with 24 than on that with 12 I.U. per gram of food.

The ages of the females at the birth of their first young averaged the same for these two diets. The average weights of the young at 28 days of age were also the same.

In length of reproductive life, number of young borne, number of young reared, percentage reared, and total weight of young at 28 days, higher records were made by the females on diet 361 than by those on diet 362; that is, these values were more favorable with 12 I.U. than with 24 I.U. of vitamin A per gram of food. This was also true of length of life (appreciably for males, but only very slightly for females). Whether these small differences are more than accidental we do not know; their chief significance is in showing that nutritional response is essentially "plateaued" at this intake level.

As in all comparable work in this laboratory, the females lived longer than the corresponding males. By comparing the data of this with those of our previous papers, already noted,

it is clearly shown that quadrupling an adequate (or minimum adequate) allowance of vitamin A may increase the normal length of life by 10 to 12%; the "useful" segment of the life cycle — from the attainment of maturity to the onset of senility — is increased also and in greater ratio. Thus it is shown that such an increase in intake of vitamin A, above the minimum adequate level, expedites development and defers old age in the same individuals. In these experiments the optimum concentration of vitamin A in the diet was at or somewhat above 12 I.U. per gram of air-dry food, or about 4 times the level usually accepted as minimum adequate.

Storage of vitamin A in the body of the rat increased with the intake level (from 3 I.U. up to 24 I.U. per gram of dry food) in both sexes and was greater at 700 than at 500 days of age, indicating that the ability to store this vitamin continues throughout middle age.

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THE EFFECTS OF VARIOUS LEVELS OF THIAMINE AND RIBOFLAVIN INTAKE UPON THE UTILIZATION OF CASEIN, SUPPLEMENTED WITH METHIONINE [1]

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The literature concerning the effects of vitamin deficiencies upon nitrogen metabolism provides some evidence of a general physiological relationship between the vitamins and the amino acids. Some of these reports indicate that subopitimum levels, or the absence, of either thiamine or riboflavin may adversely affect the digestibility and biological value of the ingested protein (Basu and Nath, '42; Kon, '31; Sure, '41; Borgstrom and Hammersten, '44; Richter and Rice, '44; and Diaz et al., '47). Furthermore, Sure and Ford ('42) found an interrelationship between thiamine and riboflavin indicating that the efficiency of riboflavin utilization was decreased in the absence of thiamine, but that the reverse was not true. The study reported herein presents data on the effects on the digestibility and biological value of casein, supplemented with methionine, of varying the levels of both thiamine and riboflavin.

EXPERIMENTAL

The nitrogen balance technique (Mitchell, '24; Mitchell and Carman, '26; Mitchell and Hamilton, '44) was used with minor modifications to measure the digestibility and bi-

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ological value of the casein. Young male rats of the Sprague-Dawley strain were used as the test animals. They were taken from litters whose mothers had received a commercial dog food [3] until the 16th day of lactation, when they were given a diet low in thiamine and riboflavin, diet I (see table 1 for composition of test diets). The weanling males (35 to 40 gm) were randomized, placed in individual metabolism cages, maintained in a room at a temperature of 78–80°F. and fed a purified diet (diet II) free of thiamine and riboflavin. Some of the rats were given a large daily supplement of thiamine (50 μg) and thus depleted only of riboflavin. Similarly, others were given 50 μg of riboflavin and depleted of thiamine. When their weights were constant for three days the rats were considered depleted of their major vitamin reserves. This occurred in about 10 to 12 days when thiamine was absent from the diet, in about 7 days when riboflavin was omitted, and in about 10 to 12 days when both vitamins were withheld. They were then fed diet III, also free of thiamine and riboflavin but containing a lower amount of protein, about 10%. In diets II, III and IV dextrose was substituted for cornstarch since it has been shown that there is less intestinal synthesis of the B-complex vitamins when dextrose is the source of carbohydrate (Guerrant and Dutcher, '34; Guerrant et al., '37; Griffith, '35; Morgan et al., '38; and Mannering et al., '44).

Mitchell's technique was modified and all groups of rats, with the exception of group 2, received diet III and the same supplement in both the 7-day experimental periods, A and C, and a protein-free ration, diet IV, in the 7-day standardizing period, B. It was necessary to do this because the usual standardizing diet containing 4% egg protein would provide appreciable amounts of the vitamins under investigation. Rats receiving the higher levels of the vitamins were given the same amount of basal diet as were those on the lower levels. The total food eaten per rat per period was recorded (see table 2). Ferric oxide was added to the food as a marker on the first and last day of each test period. Feces for one entire period were

[3] Purina dog chow.

placed in concentrated sulfuric acid in a Kjeldahl flask, digested, made to volume and sampled.

A few of the rats, used as controls, were not depleted of either vitamin and received the commercial dog food up to the time they were put on the experiment. Some of these con-

TABLE 1

Composition of experimental diets

CONSTITUENT		DIET					
		I	II	III	IV	V	VI
		gm	gm	gm	gm	gm	gm
Cornstarch		36	..	..	..	61	66.6
Dextrose		15	63	71	81	10	10
Casein, purified [1]		..	18	9.4	..	9.4	..
Casein, crude		30	..	..	..	..	..
DL-Methionine [1]		..	..	0.6	..	0.6	..
Egg, whole dried		..	..	..	..	..	4.4
Cottonseed oil		11	11	11	11	11	11
Mineral mixture [2]		4	4	4	4	4	4
Agar		2	2	2	2	2	2
Sodium chloride		1	1	1	1	1	1
Fish liver oil [3]		1	1	1	1	1	1
Vitamins:							
Alpha-tocopherol	mg	...	2.24	2.24	2.24	2.24	2.24
Ascorbic acid	mg	..	0.5	0.5	0.5	..	..
Biotin (free acid)	μg	..	2.25	2.25	2.25	..	..
Choline chloride	gm	0.15	0.25	0.25	0.25	0.15	0.15
Folic acid	μg	..	0.06	0.06	0.06	..	..
Inositol	gm	..	0.10	0.10	0.10	..	..
Nicotinic acid	mg	..	0.63	0.63	0.63	..	
Calcium pantothenate	mg	0.30	0.50	0.50	0.50	0.30	0.30
p-Aminobenzoic acid	mg	..	0.30	0.30	0.30	..	..
Pyridoxine hydrochloride	mg	0.30	0.63	0.63	0.63	0.30	0.30
Vitamin K	mg	..	0.21	0.21	0.21	..	
Riboflavin	mg	..	..	..	..	0.40	0.40
Thiamine hydrochloride	mg	..	..	..	..	0.50	0.50

[1] Vitamin test casein (14.9% N; 0.14 μg riboflavin per gram; 0.0 μg thiamine) and DL-methionine, GBI, manufactured by General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Mitchell ('44) mineral mixture No. 446.

[3] Three thousand U.S.P. XI units of vitamin A and 400 A.O.A.C. units of vitamin D per gram.

TABLE 2

The effect of various levels of thiamine and riboflavin intake upon the utilization of casein, supplemented with methionine, in diet III

GROUP NO.	VITAMIN SUPPLEMENT		PERIOD	AVERAGE PER PERIOD [1]					AVERAGE BIOLOGICAL VALUE [2] (both periods)
	Thiamine HCl	Riboflavin		Food eaten per rat	Weight of rat	Gain in weight of rat	Digestibility	Biological value	
	μg/day	*μg/day*		*gm*	*gm*	*gm*	%	%	%
	No depletion								
1	50	50	A	37	73	6	100	87	
			C	36	79	7	99	91	89
2	Diet V Mitchell		A	55	84	12	100	92	
			C	54	119	13	100	97	95
	Thiamine depleted								
3	0.00	50	A	24	..	..	..	..	
			C	..	..	..	..	..	..
4	1.25	50	A	26	62	0	100	46	
			C	29	53	0	99	61	53 [3]
5	2.50	50	A	31	66	1	99	69	
			C	33	69	5	99	82	76
6	5	50	A	38	69	6	99	88	
			C	37	74	7	97	88	88
7	10	50	A	39	74	6	99	85	
			C	39	78	8	98	89	87
8	20	50	A	39	72	9	100	92	
			C	37	78	9	100	97	95
9	50	50	A	39	74	7	99	88	
			C	37	78		97	91	90
	Riboflavin depleted								
10	50	0	A	31	53	2	99	71	
			C	28	50	1	97	44	58 [4]
11	50	1.25	A	34	63	3	100	81	
			C	32	64	3	98	81	81
12	50	2.50	A	34	55	5	99	80	
			C	33	61	4	98	82	81
13	50	5	A	35	61	6	99	90	
			C	35	68	8	98	92	91
14	50	10	A	35	64	8	100	87	
			C	35	73	8	98	90	89
15	50	20	A	35	65	8	100	96	
			C	35	72	7	98	94	95
16	50	50	A	35	61	8	99	94	
			C	35	69	8	99	94	94
	Thiamine and riboflavin depleted								
17	2.50	1.25	A	34	62	5	99	65	
			C	30	63	2	97	81	73
18	2.50	2.50	A	31	56	5	100	73	
			C	29	60	4	98	85	79
19	5	1.25	A	34	58	4	100	74	
			C	30	61	3	97	80	77
20	5	2.50	A	35	60	7	100	80	
			C	32	66	5	98	87	83

[1] Average of 4 replicates.
[2] Average of 8 determinations.
[3] Difference in means necessary for significance at a 5% level is 11.7%. See table 3.
[4] Difference in means necessary for significance at a 5% level is 10.8%. See table 4.

trol rats were placed in *group 1* and fed the maximum levels of both of the vitamins. Others, placed in *group 2*, received diet V in periods A and C and diet VI in period B. The latter were treated according to the specific directions of Mitchell and Hamilton ('44) in order to determine, under standard conditions, the biological value of the casein, supplemented with methionine. Purified diets V and VI were those proposed by Mitchell and Hamilton ('44).

Other rats depleted of thiamine were placed in *groups 3 to 9* and received one of the following thiamine hydrochloride supplements in micrograms: 0.0, 1.25, 2.5, 5.0, 10.0, 20.0, 50.0, together with 50 μg of riboflavin per day. The riboflavin-depleted rats were placed in *groups 10 to 16* and given similar levels of riboflavin with 50 μg of thiamine.

The vitamin mixture added to diets II, III and IV, table 1, was based on levels suggested by Boutwell et al. ('45) and contained 7 vitamins not included in the regular Mitchell diet. The thiamine and riboflavin supplements were in solution and this was added daily to the food.

Since facilities were inadequate, it was impossible to carry on the desirable number of replications at one time. However, a complete replicate of groups 1 through 16 was made during each of 4 experimental periods. There were two test periods in a replicate and hence 8 figures for biological value were obtained for each level of the two vitamins.

To determine whether intestinal synthesis of riboflavin was taking place, as suggested by Ziegler ('46), 2% sulfasuxidine [4] (succinylsulfathiazole, Welch and Wright, '43) was included in diets III and IV at the expense of the dextrose, and a complete replicate of the riboflavin levels was carried out. It will be noted that diets III and IV contained known amounts of para-aminobenzoic acid, biotin, folic acid and methionine — factors which Welch and Wright ('43) found effective in combatting the growth-inhibiting effect of another sulfa compound, sulfaguanidine. Since the results indicated that the sulfasuxidine caused no marked difference in growth or in biological

[4] Sulfasuxidine donated by Sharp and Dohme, Inc., Glenolden, Pa.

value, it seemed probable that intestinal synthesis of riboflavin was not taking place on this diet, containing dextrose as the carbohydrate, and the data relating to it are therefore not presented in this paper.

On the basis of the results of the first series, in which the requirement of thiamine or riboflavin was studied in the presence of adequate amounts of the other vitamin, a second series was devised in which lower amounts of each of the vitamins were used. Since the rats in the first series receiving 1.25 μg of thiamine failed to grow and developed polyneuritis, this level was not repeated; thiamine was fed at either 2.5 or 5.0 μg and riboflavin at either 1.25 or 2.50 μg. The 4 possible combinations of these levels were fed to rats that had been depleted of both thiamine and riboflavin, *groups 17 to 20*, table 2.

Seven-day fecal and urinary collections were made after a 5-day stabilization period, and nitrogen determinations on the excretions and diets followed the Kjeldahl method. Values for endogenous nitrogen for each of the experimental periods A and C were calculated by correcting the urinary nitrogen of period B for body weight and metabolic nitrogen by adjusting the fecal nitrogen of period B for food consumption.

The biological values and digestibilities were calculated by the following formulae:

$$\frac{\text{Food N} - (\text{Fecal N} - \text{Metabolic N}) - (\text{Urinary N} - \text{Endogenous N})}{\text{Food N} - (\text{Fecal N} - \text{Metabolic N})} \times 100 = \text{Biological Value};$$

$$\frac{\text{Food N} - (\text{Fecal N} - \text{Metabolic N})}{\text{Food N}} \times 100 = \text{Percentage Digestibility}.$$

RESULTS AND DISCUSSION

Data showing the effects of various levels of thiamine and riboflavin intake on the digestibility and biological value of casein, supplemented with methionine, are summarized in table 2. It is obvious that the level of thiamine and riboflavin intake did not affect the digestibility of the test protein, as all values were between 97 and 100%.

The average biological values of the test protein ranged from 53 to 95%, and these findings, though limited in number,

were treated statistically according to the methods of Snedecor ('46) and Paterson ('39). The analysis of variance of the biological values, together with the difference in means necessary

TABLE 3

Analysis of variance for data obtained on the biological value of casein, supplemented with methionine, in groups 3 to 9

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F. VALUE
Levels of thiamine	5	1810.2	13.5 [1]
Replications	3	42.2	
Error	39	134.6	
Total	47		

[1] Total exceeds the 1% point for the distribution of F. (Snedecor, '46). Standard error of difference between means $= \sqrt{\frac{134.6 \times 2}{8}} = 5.8$.

Difference in means necessary for significance =

5.8×2.70 (0.01 value of t) $= 15.7$ at 1% level.

5.8×2.02 (0.05 value of t) $= 11.7$ at 5% level.

TABLE 4

Analysis of variance for data obtained on the biological value of casein, supplemented with methionine, for groups 10 to 16

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F. VALUE
Levels of riboflavin	6	1336.1	11.7 [1]
Replications	3	145.0	1.3
Error	46	114.6	
Total	55		

[1] Exceeds the 1% point for the distribution of F. (Snedecor, '46). Standard error of differences between means $= \sqrt{\frac{114.6 \times 2}{8}} = 5.35$.

Difference in means necessary for significance =

5.35×2.69 (0.01 value of t) $= 14.4$ at 1% level.

5.35×2.01 (0.05 value of 5) $= 10.8$ at 5% level.

for significance for groups 3 to 9 and 10 to 16, are presented in tables 3 and 4 respectively.

The average biological values for most groups are slightly lower in period A than in the second period, C. Discussion of

this difference has been reviewed by the authors in another paper in this issue (Mayfield and Hedrick, '49), and has received further attention in a recent article by Allison et al. ('46).

Effect of various levels of thiamine intake upon protein utilization

The biological value of the test protein was significantly affected by the level of thiamine intake, as is shown in table 3. Further analysis of the data shows that the difference in means necessary for significance at the 1% level is 15.7 and at the 5% level is 11.7%. Using this per cent as the difference in means necessary for significance, it will be noted that changing Mitchell's basal diets V and VI, containing cornstarch, to diets III and IV, containing dextrose as the major source of carbohydrate, caused no lowering in the utilization of the test protein, since the values of 89 and 95, for control groups 1 and 2, are not significantly different (see discussion on the composition of the experimental diets). Depleting the rats of their body stores of thiamine did not affect this value, since group 9, thiamine depleted, gave a value of 90 as compared to 89 for group 1 with no depletion.

Values representing the utilization of the protein could not be determined for rats continued on a thiamine-free diet, group 3, since the animals did not survive the test periods. The two lower levels of thiamine, 1.25 and 2.50 μg per day, groups 4 and 5, permitted survival, and the biological values were lowered significantly to 53 and 76%, respectively. Rats in group 4, receiving 1.25 μg of thiamine, failed to grow and were the only ones to develop polyneuritis. The feeding of the next 4 supplements at the levels of 5, 10, 20, and 50 μg produced no significant changes, the results being 88, 87, 95 and 90%, respectively.

Changes in body weight of the rats during the experimental periods A and C, expressed as "average gain in weight," are given in table 2. Rats in group 4, receiving 1.25 μg of thiamine per day, were the only animals that did not make some gain in

weight during the test period. Hence there is no indication that the low biological values obtained in groups 4 and 5 were due to a loss of body protein.

Rats receiving 5 μg of thiamine per day, group 6, utilized their protein as efficiently as did those receiving the higher levels. Taking into consideration the amount of food consumed by the rats in this group, the daily supplement of 5 μg of thiamine was comparable to a diet containing 100 μg per 100 gm of food. It is interesting to note that this level, at which the rats made maximum utilization of the test protein, is similar to that reported by Arnold and Elvehjem ('38) for normal growth, i.e., 80 to 100 μg of thiamine hydrochloride per 100 gm of diet.

Effect of various levels of riboflavin intake upon protein utilization

The statistical treatment of the data for groups 10 to 16, as given in table 4, indicates that the biological value of the test protein was significantly affected by the level of riboflavin intake. The difference in means necessary for significance at a 1% level is 14.4 and at a 5% level is 10.8%.

Rats in group 10, table 2, depleted of riboflavin and continued on a riboflavin-free diet, lived through both experimental periods and yielded data indicating an average biological value of 58%. Since in most cases the biological values are slightly lower in period A than in C (as previously discussed), it is probably significant that as the rats in group 10 were further depleted of riboflavin in period C, they were less able to utilize their protein and a low value of 44% was obtained as compared to 71% in period A.

Using 10.8% as the difference in means necessary for significance, table 4, it will be noted that the average biological value of 81% for groups 11 and 12, receiving 1.25 and 2.50 μg of riboflavin per day, was significantly lower than that obtained when the rats received the higher levels of the vitamin. Daily supplements of 5.0 μg of riboflavin allowed the rats to utilize the test protein as efficiently (biological value of 91%)

as did the higher levels of 10, 20 and 50 μg (biological values of 89, 95 and 94%). These values are similar to those obtained with the control groups.

All rats in groups 10 to 16, table 2, showed some gain in weight during the experimental periods and, as discussed in the previous section, there is no indication that the low biological values obtained in groups 10, 11 and 12 were due to a loss of body protein.

These data suggest that a daily supplement of 5 μg of riboflavin was necessary for the maximum utilization of the test protein. This value is similar to that found for thiamine and is comparable to a diet containing 100 μg of vitamin per 100 gm.

Effect of various levels of thiamine and riboflavin intake upon protein utilization

Rats in groups 17 to 20, table 2, depleted of both thiamine and riboflavin, were given the various combination supplements of 1.25 and 2.50 μg of riboflavin and 2.50 and 5.0 μg of thiamine. When the biological value for group 17 is compared with those for corresponding groups 5 and 11, 18 with 5 and 12, 19 with 6 and 11, and 20 with 6 and 12, it will be noted that the values are rather similar and that low levels of both of the vitamins do not seem to have an additive detrimental influence on the utilization of the protein.

SUMMARY

1. The effect of various levels of thiamine and riboflavin intake upon the utilization of casein, supplemented with methionine, was studied. The nitrogen balance technique with weanling rats was used in this investigation, following the general method of Mitchell.

2. Necessary minor modifications in method did not affect the biological value of the test protein: (a) Utilization of the protein was similar when rats received dextrose in place of cornstarch as the source of carbohydrate; (b) depleting the rats of their major reserves of either thiamine or riboflavin

did not permanently affect their ability to utilize the test protein when they were given the various vitamin supplements during the experimental period.

3. The level of thiamine and riboflavin intake did not affect the digestibility of the protein. All digestibility values were between 97 and 100%.

4. Statistical analysis of the data showed that the biological value of the test protein was significantly affected by the level of thiamine or riboflavin intake.

5. Biological values for the test protein, obtained with the control groups of rats, were 89 and 95%.

6. Rats receiving daily supplements of 1.25 and 2.50 μg of thiamine hydrochloride, with all other factors optimum, were unable to utilize the protein as efficiently as did those receiving the larger amounts, and biological values were significantly lowered to 53 and 76%, respectively. Rats receiving 5.0 μg of thiamine daily utilized their protein (biological value of 88%) as efficiently as did those receiving 10, 20 and 50 μg of the vitamin (biological values of 87, 95 and 90%).

7. Groups of rats receiving daily supplements of 0, 1.25 and 2.50 μg of riboflavin did not utilize their protein as efficiently (biological values of 58, 81 and 81%) as did the control groups. At a 5.0 μg level of intake (biological value of 91%), protein utilization was as efficient as at the higher levels of 10, 20 and 50 μg of riboflavin (biological values of 87, 95 and 90%).

8. Feeding supplements of both thiamine and riboflavin at a low level did not show an additive detrimental influence on the utilization of the protein.

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THE EFFECT OF CANNING, ROASTING AND CORNING ON THE BIOLOGICAL VALUE OF THE PROTEINS OF WESTERN BEEF, FINISHED ON EITHER GRASS OR GRAIN [1]

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INTRODUCTION

Interest has developed recently in the nutritive value of protein in foods and how it may be affected by the various processing procedures. The efficiency with which a given protein supplies the nitrogen requirements of an animal is defined in terms of its biological value. An effective protein is one which is highly digestible and absorbable and which supplies the organism with adequate amounts of the amino acids which it needs. Roasting and canning may affect the physical properties of meat protein adversely by changing the linkages so that they are not as susceptible to enzymatic digestion (Hawk et al., '47). Morgan and Kern ('34) found that the biological value decreased in proportion to increase in severity of the heat treatment. The salts used in the corning process, due to their peptizing behavior, may, in contrast, increase the effect of the digestive enzymes by rendering the protein more soluble (Gortner et al., '28). However, due to the long immersion of

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the meat in the concentrated brine, unfavorable physical changes in the proteins may take place and thus decrease their digestibility.

Meat from grain-fed animals is, according to general belief, more tender and palatable than meat from grass-fed animals. Since tenderness may be correlated with digestibility, this investigation was undertaken to study, first, the effect of varying the types of feed, and second, the subsequent changes produced by canning, corning and roasting on the digestibility and biological value of beef protein.

METHODS

The meat was obtained from 17 two-year-old Hereford steers that had been on controlled feeding experiments conducted by the Department of Animal Industry. Three of them were finished on Montana grass, while groups of two, 5 and three animals were fed a barley and beet-pulp combination for the last 60, 90 and 180 days, respectively. Four steers were fed the grain mixture throughout the experimental period. After slaughtering, the meat was allowed to age for approximately 10 days in a dark room at 33–35°F.

Raw beef

The eye muscles of the 12th rib cuts were ground, dehydrated at 60°C., reground, fat-extracted and used in the experimental diets as a control for the experiment on roasted beef (Mitchell and Beadles, '37). Similarly, representative samples of raw beef round were removed from the meat to be used for canning and corning experiments.

Roasted beef

The right and left 10th-to-11th standing-rib cuts from each of the three grass-fed animals were roasted in an open pan at 163°C. (325°F.) to an internal temperature of 80°C. The outer portions were discarded and meat from the eye muscles of the cooked roasts was thoroughly mixed, ground and prepared in the same manner as the raw beef.

Canned beef

Lean beef round was cut into pieces, browned in an open pan in the oven for 30 minutes, packed hot into pint glass jars and processed at 250° F. for 85 minutes, following the recommendation of Stanley et al., of the U. S. Department of Agriculture ('42). The entire contents of the jars were ground and prepared as were the other meats.

Corned beef

Large pieces of beef round were cured for 60 days in a brine consisting of: 2 gal. water, 4 lb. NaCl, 1.5 lb. sugar, 2 oz. $NaHCO_3$, and 1 oz. $NaNO_3$. Representative samples of the corned beef, free from excess brine, were prepared in the same manner as the other meat samples. Compensation was made for the salt content of this meat when it was used in the experimental diet.

Method for determining biological value of protein

Weanling, male albino rats of the Wistar strain were used in the paired-feeding experiments according to Mitchell's nitrogen balance method (Mitchell, '24; Mitchell and Carman, '26; and Mitchell and Hamilton, '44). Each pair of litter mates was used to test the biological value of both the raw and processed samples of meat. One rat of the pair was given the raw sample in the first test period and the processed sample in the second. Four or 5 pairs composed each test group and the paired-feeding technique was used. The percentage composition of the experimental diets was as follows: Sucrose, 10; agar agar, 2; cottonseed oil, 8.5; cod liver oil, 1.5; salt mixture, 4 (Mitchell and Hamilton, '44); protein, 10 to 12 (supplied in ground, dried beef); vitamin supplement, 5 (a mixture of synthetic vitamins with starch); and enough cornstarch to make 100%. The vitamin supplement consisted, in grams, of the following: Calcium pantothenate, 0.03; choline chloride, 15.0; pyridoxine hydrochloride, 0.03; riboflavin, 0.04; thiamine hydrochloride, 0.05; these vitamins, in the amounts indicated,

were mixed with 484.85 gm of cornstarch. When the daily protein intake of the rats had been constant for 5 days, the 7-day period (A) was begun. This was followed by a period of low nitrogen intake (B), using a diet containing 4% egg protein, then by another test period (C) with the meat samples of period A reversed. Throughout these three periods the urine and feces were collected daily. Food consumption was controlled and recorded.

Nitrogen analyses of the food and excreta were made by the Kjeldahl method. Since the wastages of dietary nitrogen in digestion are contained in the fecal nitrogen, metabolic nitrogen for periods A or C is calculated by adjusting the fecal nitrogen of period B (standardizing period) for food consumed; e.g., Metabolic Nitrogen = Fecal Nitrogen (period B) $\times \frac{\text{food consumed in period A}}{\text{food consumed in period B}}$. Similarly, the endogenous nitrogen for periods A or C is determined by correcting the urinary nitrogen of period B for body weight; e.g., Endogenous Nitrogen = Urinary Nitrogen (period B) $\times \frac{\text{body weight in period A}}{\text{body weight in period B}}$ (Mitchell, '43). The biological value and digestibility were calculated from the following formulae:

$\frac{\text{Food N} - (\text{Fecal N} - \text{Metabolic N}) - (\text{Urinary N} - \text{Endogenous N})}{\text{Food N} - (\text{Fecal N} - \text{Metabolic N})} \times 100 =$ Biological value. $\frac{\text{Food N} - (\text{Fecal N} - \text{Metabolic N})}{\text{Food N}} \times 100 =$ Percentage Digestibility.

Analysis of results

The data were treated statistically according to the method of analysis of variance (Snedecor, '46).

RESULTS AND DISCUSSION

In table 1 individual metabolic data are given for 10 rats receiving raw beef round, in order to show the variations obtained in this type of experiment. The remainder of the table is composed of the averages of 4 to 5 rats for each of two periods. Data are presented showing the effects of canning, roasting and corning on the digestibility and biological value of the meat.

TABLE 1

TABLE 1

Metabolic data showing the biological value of western beef when raw, canned, roasted and corned

RATION	MEAT		PERIOD	AVERAGE WT. OF RAT	N INTAKE	URINARY N	FECAL N	ENDOGENOUS N	METABOLIC N	DIGESTIBILITY	BIOLOGICAL VALUE
	Treatment	Cut		gm	mg	mg	mg	mg	mg	%	%
Grass-fed	Raw	Round	A	90	87	35	11	12	9	98	72
			A	86	87	38	10	9	9	99	67
			A	85	87	31	14	10	8	93	74
			A	76	87	29	10	10	8	98	78
			A	82	87	33	10	10	9	98	73
										Average	73
			C	128	87	32	11	17	9	97	82
			C	124	87	28	10	16	9	99	86
			C	113	87	30	9	16	8	99	83
			C	110	87	26	9	18	8	99	91
			C	131	87	25	11	13	9	97	86
										Average	86
	Canned	Round	A	81	88	37	14	11	9	94	69
			C	120	88	32	13	15	9	94	79
Grass-fed	Raw	Rib	A	84	87	37	10	10	9	98	70
			C	128	87	28	10	16	8	98	86
	Roasted	Rib	A	88	86	35	9	11	8	99	71
			C	127	86	26	10	15	9	98	87
Grass to 60-day grain	Raw	Round	A	81	109	50	9	19	8	99	72
			C	112	123	44	9	28	8	100	88
	Corned	Round	A	77	109	55	11	20	8	97	66
			C	115	120	44	10	27	9	99	86
Grass to 90-day grain	Raw	Round	A	79	100	51	9	21	8	99	69
			C	112	113	47	10	29	8	99	85
	Corned	Round	A	78	103	55	9	20	7	98	66
			C	111	119	45	10	29	9	99	87
Grain-fed	Raw	Round	A	78	107	49	10	17	8	99	70
			C	114	121	43	10	25	9	99	85
	Corned	Round	A	75	107	52	8	16	8	99	66
			C	115	114	44	10	25	9	99	83

The biological values as calculated for each period differ considerably, but confirm the work of Mitchell ('28). Whether the value of period A or C or an average should be selected as the true biological value is open to question. This difference is interpreted by Chick et al. ('35) as a "nitrogen debt" incurred during specific protein starvation. Mitchell and Beadles ('37) suggested that the discrepancy was due to the fact that in the low nitrogen feeding period the endogenous level of nitrogen excretion had not been attained, thus increasing the biological value. More recently, Mitchell has recommended the use of a 7-day preliminary feeding period (Sahyun, '48). Differences in biological values between periods A and C may be due in part to incomplete adjustment of the animals to the experimental rations in the 5-day preliminary period used in this study.

Since this is a comparative study and the values for periods A and C vary rather widely, it would appear that the data obtained for the two periods should be treated and compared separately. The average biological values of raw beef round (73%, period A; 86%, period B) obtained in this investigation are somewhat comparable to that (76%) found by Mitchell and Beadles ('37). Morgan and Kern ('34), using the lower protein level of 7 instead of 10% and measuring the requirements for maintenance alone, obtained lower biological values for raw bottom round (67%) and found that cooking decreased these values further.

It will be noted that in period A canning lowered the average biological value of the beef protein from 73 to 69%. When the individual data were treated statistically according to the method of analysis of variance, the "F" value obtained, 3.44, was not as large as that required for significance, 5.12, in Snedecor's ('46) "F" table. However, the "F" value obtained is large enough to suggest that if more rats had been used in the study the lowered biological value of the canned meat obtained in this period might have been significant. When the data from period C were treated in the same manner it was found that the lowered value of the canned meat,

79% as compared to 86% for the raw, was significant at a 5% level with an "F" value of 9.15. Analysis of the data indicated that canning significantly lowered the digestibility of the protein from 97 and 98 to 94% in periods A and C respectively. The processes of roasting and corning did not bring about any significant change in either the biological value or digestibility.

When the data were analyzed further, no differences were found in the biological value and digestibility of the meat proteins from grain-fed and grass-fed animals. Similarly, in a previous study, the thiamine and riboflavin values of beef from grain-fed and grass-fed animals were comparable (Mayfield and Hedrick, in press).

SUMMARY

The effect of varying the type of feed and the subsequent changes produced by canning, roasting and corning on the digestibility and biological value of beef protein were studied.

The nitrogen balance technique with weanling rats was used for this investigation, following the method of Mitchell. When values for the periods were compared separately, statistical analysis of the data showed that in period C canning caused a slight but significant lowering in the biological value (86 to 79%). In period A, however, the difference (73 to 69%) was not significant. Digestibility values were significantly lowered by the canning process, from 98 to 94% in both periods. Roasting and corning did not change either the biological value or digestibility.

Beef round from grass-fed and grain-fed animals had similar biological and digestibility values.

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COMPARATIVE EFFECTIVENESS OF VITAMIN B_{12}, WHOLE LIVER SUBSTANCE AND EXTRACTS HIGH IN APA ACTIVITY, AS GROWTH PROMOTING MATERIALS FOR HYPERTHYRIOD ANIMALS [1]

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The relationship of the thyroid hormone to the nutrition of laboratory animals has been studied for some time (Drill, '43; Ershoff, '47a; Betheil, Wiebelhaus and Lardy, '47; Johnson, Hansen and Lardy, '48). It has been found both in this laboratory and by Ershoff ('47a, '47b) that rats ingesting desiccated thyroid gland require, in addition to all known vitamins, a growth promoting substance which is present in liver. The active principle of liver was found to be relatively heat stable and was not destroyed by autoclaving for one hour in solutions at pH 1, 7 or 10 (Betheil, Wiebelhaus and Lardy, '47).

In more recent work, the beneficial effects of anti-pernicious anemia (APA) extracts and of vitamin B_{12} have been noted. The similarity in properties between the growth promoting activity of liver and the APA factor indicated a possible re-

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lationship between the nutritional factor or factors required by hyperthyroid rats and the APA factor. Results are reported here which indicate that highly purified preparations of the APA factor are effective in *partially* preventing the growth retardation observed in rats fed thyroid substance. This finding may form the basis of an animal assay for a *growth promoting substance or substances* in APA concentrates. With the rat, a graded growth response is obtained with increasing amounts of the extracts equivalent to a daily intake of 0.01 to 0.1 USP units of the APA factor.

The growth promoting activity of various liver fractions and of other natural materials was also tested and is reported.

METHODS

The growth promoting activity of the materials studied was determined by the 4-week growth assay procedure using thyroid-fed rats previously described (Betheil, Wiebelhaus and Lardy, '47). Male, weanling, Sprague-Dawley strain rats weighing between 37 and 42 gm were grouped in lots (usually 6 rats) the average weights of which were equal. Negative (basal ration plus desiccated thyroid USP [2]) and positive (basal ration only) control groups were included in each experiment. Activity was determined by comparing growth on the thyroid-containing ration plus the supplement with that of the positive and negative controls. Using this growth assay the relative activity of the materials studied was usually compared with that obtained by the addition to the ration of a supplement of the 3 or 5% whole liver powder. This level of whole liver powder is insufficient to give a maximum response but it was used since it provides a sensitive estimate of the value of fractionation procedures on liver substance. It will be shown that liver powder at a 10% level completely reverses the growth inhibition caused by feeding desiccated thyroid at a level of 0.25% of the ration.

[2] Wilson.

The basal ration contained the following in parts by weight: casein [3] 22, sucrose 68.5, salts IV (Phillips and Hart, '35) 4.5, and corn oil 5. Vitamins were added to each kilogram of ration at the following levels: thiamine hydrochloride, 6 mg; riboflavin, 12 mg; nicotinic acid, 40 mg; pyridoxine hydrochloride, 6 mg; calcium pantothenate, 60 mg; choline chloride, 4 gm; *p*-aminobenzoic acid, 100 mg; biotin, 0.2 mg; folic acid, 0.5 mg; inositol, 2 gm; and 2-methyl-1, 4-naphthoquinone, 4 mg. Each animal received three drops of haliver oil USP [4] weekly. All rations were fed ad libitum.

EXPERIMENTAL RESULTS

Solubility of the growth promoting factor or factors of liver

Previous work (Betheil, Wiebelhaus and Lardy, '47) indicated the relative stability of the antithyrotoxic activity of liver. As a preliminary step in fractionation, an attempt was made to separate the active material from the bulk of the liver substance. Whole liver powder was repeatedly extracted with the following solvents: 60% aqueous ethanol, 95% ethanol, 1:1 ethanol-diethyl ether, chloroform. Extraction was continued with each respective solvent until the solvent layer contained no colored material. Chloroform extracts of whole liver substance were devoid of activity but each of the other solvent combinations tried removed some active material. In all cases the residue contained much of the active principle. Water extraction was likewise found to remove only *a part* of the activity. To determine whether the active material could be rendered soluble, water extraction was tried after autoclaving whole liver substance at pH 10 (Betheil, Wiebelhaus and Lardy, '47). The alkaline autoclaved whole liver powder was extracted with chloroform and the biologically inactive extract discarded. The residue was dried and powdered and then extracted repeatedly with distilled water at pH 6.8. The

[3] Vitamin test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Abbott.

combined water extracts were cleared in a Sharples centrifuge to remove all suspended material and were concentrated to dryness under reduced pressure. The results of the feeding experiments are shown under experiment A in table 1. It is evident that only a part of the activity had been brought into solution and that the residue contained the bulk of the active material.

Variation in water solubility of the growth factor or factors is indicated by the relative activity of various batches of ex-

TABLE 1

Growth promoting activity of natural materials and solubility of the liver factor

SUPPLEMENT TO BASAL RATION + THYROID [1]	AVE. WT. AT 4 WEEKS		
	Exp. A	Exp. B	Exp. C
	gm	*gm*	*gm*
1. Positive control — no thyroid substance	211 ± 15.4 [2]	167 ± 23.2	183 ± 7.2
2. Negative control (basal ration + thyroid), no supplement	133 ± 13.5	122 ± 17.8	123 ± 6.8
3. 3% whole liver powder	191 ± 7.0		162 ± 1.0
4. Alkaline autoclaved W.L.P.[3]	168 ± 9.1		
5. Water extract of $CHCl_3$-extracted 4	148 ± 7.9		
6. Residue from 5	173 ± 10.3		
7. 2% Abbott liver extract powder 1:12	173 ± 11.5		
8. 1.5% liver powder 1:20 (Wilson)			145 ± 19.1
9. 1.0% liver powder 1:20 (Wilson)			141 ± 12.8
10. 0.5% liver powder 1:20 (Wilson)			133 ± 16.9
11. 1% skim milk powder		130 ± 10.3	
12. 10% skim milk powder		127 ± 11.8	
13. 1% dehydrated canned tomatoes		131 ± 14.8	
14. 10% dehydrated canned tomatoes		149 ± 12.6	

[1] Desiccated thyroid fed at a level of 0.38% in experiment C, at 0.25% in experiments A and B. Six rats per group.

[2] Standard deviation.

[3] W.L.P. = Whole liver powder. All liver fractions were fed at concentrations equivalent to 3% whole liver powder.

tracts from fresh liver.[5] One lot was found to be as active as whole liver substance (Betheil, Wiebelhaus and Lardy, '47). Since then a second lot has been found devoid of activity. Ershoff ('47b) has stated that liver powder 1:20 had little or no antithyrotoxic activity. Results obtained in this laboratory with a third lot of liver powder 1:20 are described under experiment C, table 1. An extract from another source [6] was found only partially effective (group 7, table 1). It seems possible that variations in time between slaughter and extraction, with corresponding variations in autolysis, may be responsible for these wide differences in activity.

Growth promoting activity of anti-pernicious anemia preparations for hyperthyroid animals

The growth promoting material in liver resembles the APA factor in thermo-stability, in resistance to acid, and, to some extent, in its partial solubility in aqueous alcoholic solutions. In earlier work Sure and Buchanan ('37) showed that a liver extract used in the treatment of pernicious anemia had a beneficial effect on hyperthyroid animals, and attributed this effect to its content of B vitamins. Recently Jaffe and Elvehjem ('47) and Nichol et al. ('47) have reported a growth stimulating effect of APA preparation when fed to animals receiving a corn-soybean ration.

It has been found that purified liver extracts rich in APA activity exhibited growth promoting activity when injected into rats receiving desiccated thyroid substance in their ration.

In a preliminary experiment, the intraperitoneal injection of one USP unit of APA liver extract [7] per day into rats which had been fed a ration containing 0.38% desiccated thyroid USP for 5 weeks resulted in growth stimuation. The growth curves of these animals had previously reached a plateau and a loss in weight had begun. The liver extract immediately arrested

[5] Liver powder 1:20, Wilson.

[6] See footnote 4, page 497.

[7] Reticulogen No. 360, Lilly, 20 USP units per milliliter.

TABLE 2

Growth promoting activity of anti-pernicious anemia liver extracts

SUPPLEMENTS TO BASAL RATION + THYROID [1]	AVERAGE WEIGHT AT 4 WEEKS				
	Exp. A	Exp. B		Exp. C	Exp. D
	gm	*gm*		*gm*	*gm*
1. Positive control — no thyroid substance	171 ± 16.5 [2]	211 ± 15.4		171 ± 8.7	183 ± 20.8
2. Negative control (basal ration + thyroid), no supplement	124 ± 17.6	133 ± 13.5		130 ± 6.7	147 ± 4.5
3. 3% whole liver powder	158 ± 9.5	191 ± 7.0		159 ± 7.8	168 ± 1.2
4. 10% whole liver powder					188 ± 17.4
5. 300 μg vitamin B_{12} intraperitoneally/day		133 ± 10.1			
		Lilly product	Sharp and Dohme product	Lilly product	Sharp and Dohme product
6. APA concentrate [3] 0.005 units/day				131 ± 14.3	130 ± 13.1
7. APA concentrate 0.01 units/day				136 ± 10.5	133 ± 14.9
8. APA concentrate 0.025 units/day				144 ± 3.3	136 ± 9.0
9. APA concentrate 0.05 units/day		150 ± 14.1	152 ± 8.3	158 ± 8.7	139 ± 7.8
10. APA concentrate 0.1 units/day		150 ± 8.0	164 ± 13.0	143 ± 7.2	152 ± 9.1
11. APA concentrate 0.5 units/day		168 ± 16.9	158 ± 4.0		
12. APA concentrate 1.0 units/day	149 ± 13.4	157 ± 9.0	161 ± 8.4		
13. APA concentrate 2.0 units/day		162 ± 10.6	160 ± 4.3		
14. APA concentrate 1.0 units/day (4th week only)	137 ± 16.5				
15. Formylfolic acid, 100 μg/day.					147 ± 19.2

[1] Desiccated thyroid fed at 0.38% of the ration in experiment A, at 0.25% in experiments B, C and D. Six rats per group.

[2] Standard deviation.

[3] All APA concentrates were administered by intraperitoneal injection.

the weight loss and growth was resumed. A control group receiving only the thyroid containing ration continued to lose weight during the two-week experimental period.

The results of more extensive studies are shown in table 2. Preparations containing APA activity were almost as effective in overcoming growth retardation as was 3% whole liver substance. APA preparations were very effective when given concurrently with desiccated thyroid, and also elicited a growth response when given after thyroid had been fed for three weeks (experiment A, table 2).

In another experiment the effect of Reticulogen on recovery from thyrotoxicosis was studied. Male rats were kept on a thyroid containing ration (0.25% desiccated thyroid) for 7 weeks. Very few of the animals survived. Those which did were divided into two groups, equal with respect to average weight, of 6 animals each. All animals were losing weight at that time. The two groups were placed on the basal ration and one group was supplemented with one unit of Reticulogen per day intraperitoneally. All animals grew after removal of desiccated thyroid from the ration. However, Reticulogen-supplemented animals grew better during the first two weeks of the recovery period, averaging a 70 gm gain, while the unsupplemented group gained only 57 gm during this period. After three weeks the rats in all groups averaged the same in weight. Apparently this animal can rapidly readjust itself once the stress induced by thyroid administration is removed. After three weeks on a thyroid-free ration, the APA preparation had no effect on growth.

To determine whether the growth promoting activity of APA preparations paralleled the anti-pernicious anemia potency of the material, graded levels of two different preparations [8] were administered intraperitoneally to animals receiving the thyroid-containing ration. A group receiving 300 μg of thiamine was included in the experiment since Retic-

[8] Lilly Reticulogen and Sharp and Dohme Refined Liver Extract (No. 2505, 15 USP units/ml) were used.

ulogen contains added thiamine. All dilutions of liver extracts were made with physiological saline.

The results are shown under experiments B and C, table 2. Supplementation with thiamine had no effect. The maximum effect was obtained with levels of liver extract between 0.05 and 0.5 USP unit. At all higher levels both preparations gave approximately the same growth stimulation over that of the negative control.

Experiment C, table 2, shows the effect on growth of lower levels of the APA preparations. The extracts used in this experiment were from different batches than those used in 2A. From the data it appears that a maximum response is obtained with approximately 0.1 unit of liver extract.

Effect of liver extracts on thyrotoxic rabbits

To determine whether the antithyrotoxic effect of APA preparations could be observed in animals other than the rat, an experiment was conducted with rabbits injected with thyroxine. Weanling rabbits from a commercial breeder were fed the following basal ration (parts by weight) for a period of 6 weeks before beginning treatment: ground oats, 11; ground yellow corn, 36; soybean meal, 46; corn oil, 5; cystine, 0.3; $CaHPO_4$ 0.92, $CaCO_3$ 0.6, NaCl (iodized) 0.44, $MnSO_4 \cdot 4H_2O$ 0.04. Vitamins were added at the levels used in the purified ration described above. The ration was pelleted before use.

Thyroxine was administered intraperitoneally at the following rate: first week, one injection of 1 mg/kg; second week, 1 mg/kg on alternate days; thereafter injections of 0.5 mg/kg were made every third day for 4 additional weeks. At the beginning of the third week the rabbits were divided into two groups of equal size and with equal distribution of sexes. One group of 7 animals received a purified liver extract [9] intraperitoneally at a level of two USP units per day. The experiment was terminated after 4 weeks of Reticulogen

[9] Reticulogen, Lilly.

supplementation. The liver extract strikingly improved the survival of these thyrotoxic rabbits. Only one animal in the control group survived the 6-week period of thyroxine administration. In the group given liver extract, one rabbit died on the second day of therapy while the other 6 lived through the experimental period. Thyroxine administration caused a growth retardation in all animals and liver extract was not appreciably effective in reversing the retardation. Positive control animals not receiving thyroxine continued to grow well and all survived. Subsequent to our original finding of the beneficial effect of APA extracts on thyrotoxic rats. Robblee, Nichol, Cravens, Elvehjem and Halpin ('48) obtained similar results with chicks fed thyroid active materials.

Experiments with folic acid

The work of Jacobson and Good ('47) suggested that folic acid may be a precursor of the APA factor. An attempt was therefore made to determine whether the "intrinsic" factor of the normal stomach could convert folic acid into an antithyrotoxic material. Folic acid (1 mg/ml) was incubated with a hog stomach preparation [10] and, in a second experiment, with fresh human gastric juice (pH 1.7). The enzyme solutions were adjusted to pH 7.0 with NaOH and incubated for 48 hours at 37°C. Microbiological assays [11] with *Streptococcus faecalis* (American Type Culture Collection No. 8043) demonstrated that no significant destruction of folic acid had occurred. The enzyme-treated folic acid was assayed (100 μg/day) with thyroid-fed rats as described above. Neither the incubated mixture nor the original enzyme materials had any beneficial effect on the growth of thyrotoxic rats. Formylfolic acid prepared by the method of Gordon et al. ('48) was likewise found to have no beneficial effect on rats fed a thyroid-containing ration (experiment D, table 2).

[10] Ventriculin, Parke Davis.

[11] Kindly done by Dr. O. E. Olson and Mr. E. Fager.

Other sources of the antithyrotoxic factor or factors

Previous work (Betheil, Wiebelhaus and Lardy, '47) had indicated the presence of some antithyrotoxic substance in yeast in addition to the known vitamins. Results of assays of other natural materials are included in table 1. Skim milk powder was a poor source of the factor or factors, while canned tomatoes exhibited some growth promoting activity. In another experiment a preparation of fish solubles was found to be almost as active in promoting the growth of thyrotoxic rats as was dried whole liver substance. Nichol et al. ('47) have reported a growth stimulation due to fish soluble components with chicks fed a supplemented natural ration. In our studies with the rat no source of the factor has proved more potent than dried whole liver substance. In two experiments 10% bovine blood fibrin was inactive.

The effect of vitamin B_{12} on the hyperthyroid rat

With the isolation of crystalline vitamin B_{12} from liver (Rickes et al., '48) and the demonstration of its potent activity in eliciting hemopoietic responses in pernicious anemia patients (West, '48), it was of interest to determine whether this substance possessed growth promoting activity for the hyperthyroid rat. Ott and co-workers ('48) have recently reported the growth stimulatory activity of vitamin B_{12} for chicks.

Two types of preparations [12] were available. One was the pure crystalline material, the other a concentrate containing 2 mg vitamin B_{12} per pound in a mixture of soybean flour and charcoal. Crystalline vitamin B_{12} was administered by intraperitoneal injection, while the concentrate was added to the thyroid-containing ration. Supplements were administered for 42 days. The results are shown in table 3. It can be seen that vitamin B_{12} had pronounced growth promoting properties

[12] We are indebted to Dr. D. F. Green of Merck and Company, Inc., for generously making these preparations available.

at the levels used (0.2 μg intraperitoneally daily and 30 μg per kilogram of ration). In an earlier experiment, lower levels were found to be much less effective. Since an optimum amount of liver extract gave no better growth than vitamin B_{12}, it seems that all or most of the growth promoting activity of APA liver extracts resides in their vitamin B_{12} content. Con-

TABLE 3

Effect of vitamin B_{12} on the growth of hyperthyroid rats

LOT NO.[1]	REGIMEN	AVERAGE WEIGHT	
		26 days	42 days
		gm	*gm*
1	Basal ration	191 ± 11.1[2] (8)[3]	281 ± 19.9 (8)
2	Basal + 0.25% desiccated thyroid USP	149 ± 18.5 (6)	183 ± 21.1 (4)
3	2 + 0.2 μg vitamin B_{12} injected intraperitoneally daily	173 ± 12.1 (8)	222 ± 19.9 (7)
4	2 + 0.10 unit Reticulogen (Lilly) injected intraperitoneally daily	166 ± 9.9 (8)	213 ± 11.3 (6)
5	2 + 5% whole liver powder in the ration	180 ± 12.4 (8)	247 ± 13.7 (6)
6	2 + 30 μg vitamin B_{12} concentrate per kg of ration	163 ± 14.5 (8)	229 ± 22.2 (6)

[1] Eight rats per lot.
[2] Standard deviation.
[3] Number of animals surviving at that time.

versely, it appears therefore that the vitamin B_{12} requirement of the hyperthyroid rat lies between approximately 0.1 and 0.2 μg per day when the material is administered parenterally.

Iodinated casein and thyrotoxicosis

The possibility was investigated of producing the hyperthyroid syndrome in rats with a substance more readily available than desiccated thyroid. Iodinated casein[13] was fed at

[13] Protamone, Cerophyl Laboratories, Inc., Kansas City, Missouri.

various level to male rats fed the purified diet, with the results shown in table 4; a graded retardation of growth was obtained with increasing levels. Although it is much less effective than thyroid substance when fed at levels equal with respect to iodine supply, iodinated casein appears to be a satisfactory agent for the production of experimental hyperthyroidism.

TABLE 4

Growth retardation in rats: iodinated casein compared with desiccated thyroid

SUPPLEMENT TO BASAL RATION	AVERAGE WT. AT 4 WEEKS [1]
	gm
None	189 ± 11.0 [2]
0.38% desiccated thyroid	119 ± 5.6
0.03% iodinated casein	163 ± 12.7
0.06% iodinated casein	150 ± 10.8
0.10% iodinated casein	149 ± 7.8
0.20% iodinated casein	133 ± 4.2
0.30% iodinated casein	112 ± 9.9

[1] Three rats per lot.
[2] Standard deviation.

DISCUSSION

The results obtained in the present study demonstrate that in thyrotoxicosis rats require one or more growth factors which are not needed by normal rats fed highly purified diets. Purified liver extracts rich in the APA factor are a good source of this growth factor. As little as 0.0005 ml (about 50 μg dry matter) daily produced an appreciable response (table 2). The thyrotoxicosis produced by the thyroid-containing ration is not accompanied by an anemia. Hemoglobin levels of animals showing the typical growth depression are normal. It may well be that the growth response reported here is due to some constituent in the liver extract other than the APA factor. However, it has been found that liver fractions containing less APA activity on a dry weight basis were also less active in supporting the growth of hyperthyroid rats. If this growth stimulating factor is the same as that required for

blood cell maturation in human beings, these observations may be the basis for an animal assay for the APA factor. The graded growth response obtained with increasing doses of purified liver extracts (table 2) indicates the possibility of such an assay.

From evidence available at present it cannot be definitely stated whether or not vitamin B_{12} and the anti-pernicious anemia factor are identical. Further clinical testing will be required to determine whether the crystalline material will cure the neurological as well as hematological lesions which are typical of Addisonian pernicious anemia. Vitamin B_{12} is effective in ameliorating the growth retardation which results from the feeding of desiccated thyroid (table 3). The growth promoting activity of vitamin B_{12} in adequate amounts is of the same order of magnitude as that of APA extracts, and a close relationship between the two materials in the nutrition of the rat is indicated. The above data (table 3) definitely show a requirement for vitamin B_{12} in the nutrition of the hyperthyroid rat. The normal rat probably also requires the vitamin but the hyperthyroid animal allows a clearer demonstration of this need.

While a number of natural materials — tomatoes, fish soluble components and yeast — have been found to promote the growth of thyroid-fed rats, *none of these is as effective as liver*. In view of the fact that the pernicious anemia patient has difficulty in consuming sufficient liver to supply his needs for the APA factor, it is not surprising that these other *possible* sources of the factor have been overlooked.

There is some evidence of the dual nature of the growth factor present in whole liver powder. The solvent fractionation experiments presently reported indicate that in spite of exhaustive extraction of the liver powder only a part of the activity can be brought into solution. The possible occurrence of a bound form of the growth factor is indicated.

In some experiments (table 2) whole liver gave a greater growth response than purified extracts. Regardless of the number of factors required by the rat fed thyroxine-containing

rations, the technique just described should be well suited for studying additional growth promoting substances. The basis for this view has been previously discussed in detail (Betheil, Wiebelhaus and Lardy, '47).

SUMMARY

1. Rats fed thyroid-containing rations require for growth a factor or factors present in liver, fish soluble components and tomatoes. Skim milk and fibrin contain little or none of this factor.

2. Highly purified liver extracts rich in the anti-pernicious anemia principle carry the factor.

3. APA preparations had a significant effect on longevity when injected into hyperthyroid rabbits.

4. The significance of these observations in the development of an assay for the APA factor is noted.

5. Vitamin B_{12} was found to be a growth factor for the hyperthyroid rat.

6. Treatment of folic acid with good sources of the "intrinsic factor" did not result in the formation of a growth promoting substance. "Formylfolic acid" was likewise inactive in our assay.

7. Iodinated casein may be used to produce the thyrotoxic syndrome.

8. Exhaustive extraction of whole liver powder with 60% ethanol, 95% ethanol, 1:1 ethanol: ether or water brought only part of the active material into solution. These observations as well as others indicate the possible dual nature of the material which, in addition to the known vitamins, supports the growth of animals fed thyroxine-containing rations.

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THE SULFUR AMINO ACID REQUIREMENT OF THE INFANT[1]

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ONE FIGURE

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In our studies on the amino acid requirements of the adult (Albanese and associates, '43), we found that subjects on a methionine-deficient diet developed negative N balances which were promptly abolished by the addition of a methionine supplement, and concluded therefore that methionine is a human dietary essential. The role of cystine, however, remained in doubt, for parallel experiments with a cystine-deficient diet gave equivocal results in regard to nitrogen balance. This doubt was resolved by a subsequent investigation (Albanese and co-workers, '44) in which it was found that two healthy male subjects who developed negative nitrogen balances on a diet deficient in both sulfur amino acids (methionine and cystine) were restored to nitrogen equilibrium with a supplement to the doubly deficient diet of 18.8 mg of DL-methionine per kilogram of body weight. How far this intake is above the minimum methionine requirement could not be stated because various levels of supply below this amount were not tested.

With this information as a rough base, however, and the known fact that on a body weight basis the nitrogen require-

[1] The work described in this report was supported by grants from the National Livestock and Meat Board, Mead Johnson and Company, and National Dairy Products, Inc.

ments of the infant are about 5 times greater than those of the adult, it might be deduced that 5 times this adult value, or 94 mg of methionine per kilogram should provide the infant with more than an adequate supply of the sulfur amino acids. Nevertheless, the desirability of determining this requirement by direct measurements (which are reported here) rather than by inference is pointed up (a) by the claim that sulfur amino acids may constitute a limiting dietary factor in cow's milk formulas and (b) by our assay of isoleucine requirements (Albanese et al., '48) which indicated that infant needs for the individual amino acids do not always follow the amino acid composition of the milk proteins.

METHODS AND DIETS

In this as in previous investigations (Albanese et al., '48), the requirement of an essential amino acid was estimated on the basis of the amount needed to restore to normal physiological levels the nitrogen retention and rate of body weight gain of subjects previously maintained on a deficient diet. Previous experiences indicated the desirability of the following regimen: For the week preceding the deficient period the subjects were maintained on a "complete" diet, i.e., the deficient diet supplemented with cystine and methionine. The deficient diet was fed for the following three weeks to all 5 subjects. Subsequently the diet of three subjects was supplemented with L-methionine in graded amounts to a maximum of 3% of the protein intake. At the same time the diet of the other two subjects was supplemented with 1% L-cystine alone and then with 1% L-cystine and increasing amounts of L-methionine. Seven-day intervals elapsed between all dietary changes tested.

The observations reported here were made on normal healthy male infants who were given the purified diets in 5 feedings daily at the rate of approximately 100 cal. per kilogram of body weight and 500 mg of ascorbic acid together with 15 drops of oleum percomorphum daily. As noted previously, the diet periods were of 7 days' duration and were consecutive, but collections of excreta were omitted on week-ends. The sub-

TABLE 1

Composition of diets

(All diets fed at the rate of 100 cal. and 3.5 gm of protein per kilogram per day)

COMPONENTS	DIET							
	A	B	C	D	E	F	G	H
	gm	gm	gm	gm	gm	gm	gm	gm
Oxidized casein hydrolysate [1]	3.30	3.44	3.42	3.40	3.38	3.40	3.38	3.36
L-Tryptophane	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
L-Cystine	0.04	0.00	0.00	0.00	0.00	0.04	0.04	0.04
L-Methionine	0.10	0.00	0.02	0.04	0.06	0.00	0.02	0.04
Brewers' yeast [2]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Olive oil	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Dextri-maltose no. 2	9.60	9.60	9.60	9.60	9.60	9.60	9.60	9.60
Arrowroot starch	2.30	2.30	2.30	2.30	2.30	2.30	2.30	2.30
Salt mixture [3]	1.60	1.60	1.60	1.60	1.60	1.60	1.60	1.60
Water	78.00	78.00	78.00	78.00	78.00	78.00	78.00	78.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Estimated L-cystine content (mg)	50	10	10	10	10	50	50	50
Estimated L-methionine content (mg)	125	25	45	65	85	25	45	65

[1] N × 6.25 = grams of protein containing 0.10% of cystine and 0.28% of methionine by chemical analysis.

[2] Kindly supplied by Mead Johnson and Company and found by chemical analysis to contain 1.3% of cystine and 2.0% of methionine.

[3] The salt mixture employed had the following composition (measured in grams): $FeSO_4$ 0.9, NaCl 6, Ca gluconate 48, $Ca(OH)_2$ 12, KH_2PO_4 7, KCl 6, and MgO 0.1.

jects were partially immobilized by specially designed abdominal supports, which also held the urinary adapters in place. Twenty-four hour urine specimens were collected in bottles containing 10 ml of 15% (by volume) HCl and 1 ml of 10% alcoholic thymol. The feces were collected in 19 cm porcelain evaporating dishes which were kept in place by a properly shaped excavation in the mattress, and the daily stools for each period were accumulated under refrigeration in wide-mouth jars containing 200 ml of 70% alcohol. The subjects were weighed daily during the course of the experiment.

The composition of the diets used in this study is shown in table 1. The diets provided approximately 100 cal. per 100 gm, the percentage caloric distribution being as follows: Protein, 14; fat, 36; carbohydrate, 50. The protein moiety of the sulfur amino acid-deficient diet was prepared by tryptophan reinforcement of a hydrogen peroxide oxidized hydrolysate of casein (as described by Albanese et al., '44) which was found by chemical analysis to contain 0.28% of methionine and 0.10% of cystine. The protein equivalent of the final product was estimated as N $\times$ 6.25 and it was supplemented with 1.5% of L-tryptophan to replace that which was destroyed during acid hydrolysis. The cystine and methionine supplements were added to the diet as shown in table 1. Owing to uncertainties regarding the complete human requirements of B complex vitamins, brewers' yeast was employed instead of a mixture of the synthetically available vitamins. The quantities of cystine and methionine derived from this source were found by chemical analyses to be 6.5 and 10.0 mg, respectively, per gram. Thus, the amounts of the sulfur amino acids per kilogram of infant body weight provided by the diets can be estimated roughly (table 1). The final nitrogen content of each batch of diet was determined by micro-Kjeldahl analysis.

The data on nitrogen retention were calculated from the results of nitrogen determinations of the 24-hour urine collections, analyses of the pooled feces from each period, and computations of the daily N intake based on food consumption records and the known nitrogen content of the diets.

TABLE 2

Effect of dietary methionine on the nitrogen retention, body weight and blood proteins of the infant

INITIAL AGE AND WEIGHT OF SUBJECT	CATEGORY OF INTEREST	DIET							
		A	B	B	B	C	D	E	A
	Cystine intake (mg/kg/day)	50	15	15	15	15	15	15	50
	Methionine intake (mg/kg/day)	125	25	25	25	45	65	85	125
D.C.,	N intake (gm/day)	3.30	3.26	3.14	3.10	3.14	3.14	3.14	3.24
male,	N retention (mg/kg/day)	106	93	53	28	68	55	90	120
11 mos.	Weight change (gm/day)	+24	+14	−10	−7	0	+7	+20	+19
7.193 kg	Total plasma proteins (gm%)	6.45	6.35	6.25	6.19	5.76	5.12	5.38	5.40
	Hemoglobin (gm%)	10.40	9.90	9.90	10.80	10.20	10.60	11.10	11.10
A.H.,	N intake (gm/day)	4.55	4.28	4.18	4.08	4.18	4.18	4.18	4.14
male,	N retention (mg/kg/day)	116	82	77	47	72	49	98	110
11 mos.	Weight change (gm/day)	+20	−35	+4	+4	0	0	+17	+22
7.346 kg	Total plasma proteins (gm%)	6.11	6.68	6.92	5.60	6.13	5.73	5.82	6.80
	Hemoglobin (gm%)	11.00	10.60	10.50	11.10	10.60	10.80	10.50	11.60
R.R.,	N intake (gm/day)	1.78	1.76	1.78	1.79	1.75	1.80	1.78	1.78
male,	N retention (mg/kg/day)	96	14	17	−41	30	−6	99	112
4 mos.	Weight change (gm/day)	+24	−14	−35	−24	0	−13	+14	+27
3.538 kg	Total plasma proteins (gm%)	5.85	6.12	5.66	6.06	5.32	5.82	5.72	6.13
	Hemoglobin (gm%)	10.40	9.60	8.90	9.80	9.80	9.40	10.30	10.80

Blood samples were collected over lithium oxalate by vena puncture on the last day of each diet period. The hemoglobin concentration of these specimens was determined colorimetrically in the Klett-Summerson photoelectric colorimeter. The total plasma proteins, albumin, globulin and non-protein N were determined by the procedure described by some of the present authors (Albanese, Irby and Saur, '46).

RESULTS AND DISCUSSION

The results obtained in the first experiment (table 2) show that the feeding of the doubly deficient diet B caused a prompt and sustained fall of the daily weight gain and nitrogen retention below the control levels of all three subjects. In a growing organism such as the infant, a drop from the nitrogen retention values characteristic of the individual must be given the same interpretation as the establishment of a negative N balance in the adult, namely, that diet B is lacking in essential dietary components. This inference is supported by the concomitant decrements in rate of weight gain. It will be seen in table 2 that following the deficient period all three subjects were given in turn diets C, D, E and A, which provided the infants with 45, 65, 85 and 125 mg of L-methionine per kilogram of body weight, respectively. Examination of the resulting data discloses that the nitrogen retention and weight coefficients of the subjects were restored to normal levels by diet E but not by diets C and D, and the values of these criteria attained with diet E were not augmented by the refeeding of control diet A. From these observations it may be deduced that the sulfur amino acid needs of the infant can be adequately met by an intake of 85 mg of L-methionine and 15 mg of L-cystine per kilogram per day.

In order to assess the dispensability of cystine and its sparing action on the methionine requirements, a second experiment designed to elucidate both of these points was performed. In this experiment two subjects (table 3) were fed the cystine-supplemented diet F for one week following the three weeks of the doubly deficient regimen. And, since the cystine supple-

TABLE 3

Effect of dietary cystine and methionine on nitrogen retention and body weight and blood proteins of the infant

INITIAL AGE AND WEIGHT OF SUBJECT	CATEGORY OF INTEREST	DIET							
		A	B	B	B	F	G	H	A
	Cystine intake (mg/kg/day)	50	15	15	15	50	50	50	50
	Methionine intake (mg/kg/day)	125	25	25	25	25	45	65	125
R.M.,	N intake (gm/day)	2.90	2.80	2.90	2.90	2.90	2.90	2.90	2.90
male,	N retention (mg/kg/day)	102	33	35	13	37	82	109	109
6 mos.	Weight change (gm/day)	+21	−21	−14	−14	−21	−13	+18	+20
5.167 kg	Total plasma proteins (gm%)	5.70	6.38	6.23	6.43	6.26	4.82	5.42	5.83
	Hemoglobin (gm%)	11.20	8.20	8.70	9.40	10.10	10.20	9.90	10.90
H.G.,	N intake (gm/day)	3.63	3.83	3.52	3.54	3.54	3.54	3.54	3.54
male,	N retention (mg/kg/day)	111	92	47	16	41	99	128	120
8 mos.	Weight change (gm/day)	+14	−31	−21	0	−7	+14	+13	+14
7.560 kg	Total plasma proteins (gm%)	6.55	6.71	6.67	6.70	6.72	6.67	6.85	6.83
	Hemoglobin (gm%)	9.60	10.10	9.90	10.60	8.50	10.40	9.90	11.10

ment (35 mg per kilogram) failed to raise the nitrogen retention and weight gain induced by deficient diet B, it is concluded that cystine is not a dietary essential for the infant. In the subsequent week the cystine-supplemented diet F reinforced with 20 mg of L-methionine per kilogram (diet G) was fed and some improvement in the growth function was noted. However, the complete return to normal physiological N retention and weight gain values was achieved only in the following week when diet H was fed, which provided the infants with a total of 65 mg of methionine and 50 mg of cystine per kilogram of body weight. That these amounts of the sulfur amino acids were optimum is suggested by the observation that the growth criteria were not augmented by the feeding of control diet A in the last week of the experiment.

Since diet H in this experiment and diet E of the previous experiment appear to be equally capable of restoring N retention and weight gains to normal levels by subtraction of the amounts of cystine and methionine contained, respectively, in the diets, it can be deduced that in the presence of adequate quantities of methionine, 35 mg of cystine can spare approximately 20 mg of methionine. In terms of amino acid sulfur, 9 mg of cystine S are equivalent to about 4 mg of methionine S. This computation indicates that about 22% of the methionine requirement of the infant can be met by cystine, a figure which compares favorably with the 16% found by Womak and Rose ('41) for the immature rat.

In considering some of the implications of this study, it occurred to us that the results presented here might be of value in clarifying the clinical claim that the nutritive virtues of human milk over cow's milk are due to the higher sulfur amino acid content of the former. On the basis of calculations from the available data on the sulfur amino acid content of breast milk and various estimates of the intake of breast milk by infants of different ages (fig. 1), it would appear that even on the most liberal intake estimates breast milk provides a very meager margin of safety during the early months of life, and that it does not provide an adequate quantity of the sulfur

amino acids in the latter part of the first year. On the same basis cow's milk formulas, on the other hand, seem to provide adequate amounts of sulfur amino acids. These calculations support the view (albeit tentative since the calculations are based on relatively short-term experiments) that the claimed superior biological value of breast milk cannot be due in any great measure to the sulfur amino acid content of this food.

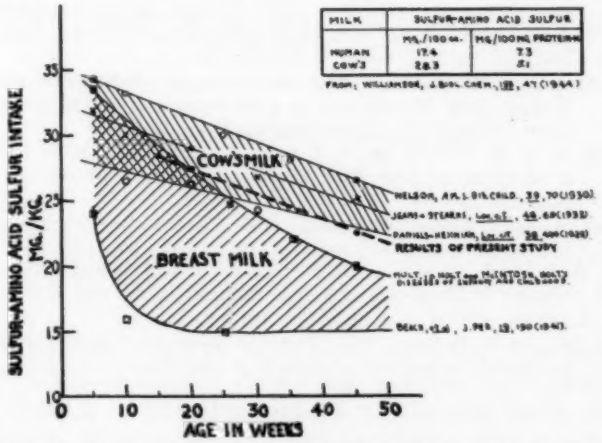

Fig. 1 Schematic comparison of the sulfur-containing amino acid sulfur provided by cow's milk formulas and by human milk with reference to the measured requirements of the infant.

Some support for this view can also be derived from the data reported by Gordon and his associates ('47), which point to the conclusion that mixtures of cow's milk will produce larger gains in weight in premature infants than will human milk.

SUMMARY

The sulfur amino acid requirements of 5 normal male infants (4–11 months of age) were studied. In three infants who received no cystine supplements the nitrogen retention and body weight gains were restored to normal levels by an intake of 85 mg of L-methionine per kilogram of body weight. In two

infants who received a 1% L-cystine supplement the nitrogen retention and weight change were restored to normal values by an intake of 65 mg of L-methionine per kilogram of body weight. The implications of these findings are discussed in reference to the nutritional virtues of human and cow's milk.

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TRYPTOPHAN AND NICOTINAMIDE IN THE NUTRITION OF THE ANIMAL MICROORGANISM, TETRAHYMENA [1]

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The many reports which have appeared in the literature during the past few years leave little doubt that the higher animals (rat, pig, dog, human) develop niacin deficiencies on diets low in tryptophan, and that the deficiency can be overcome by the addition of either niacin or tryptophan to the diet. Investigators have not been in agreement, however, regarding the site of niacin synthesis. It has been pointed out a number of times that the intestinal microflora may be responsible for the conversion of tryptophan to niacin rather than the animal *per se*. Recently Mitchell, Nyc and Owen ('48) produced strong evidence that the rat is capable of niacin synthesis when they found that the parenteral administration of hydroxyanthranilic acid, an intermediate in the chain of reactions from tryptophan to niacin in mutant Neurospora (Mitchell and Nyc, '48; Bonner, '48), resulted in a greatly increased N^1-methylnicotinamide excretion. On the other hand, Ellinger and Kader ('47) have demonstrated the importance of the microflora in niacin synthesis.

It seemed important to us to examine the problem of niacin synthesis in a given animal under conditions where the results could be unequivocally assigned to the responsibile mecha-

[1] Aided by a grant from the William-Waterman Fund for the Combat of Dietary Diseases.

nisms, within the animal, ruling out all possibilities of contaminating organisms. It has been possible to examine at the same time the effects of corn extract for a possible "pellagragenic agent." The results of these experiments, together with the short report by Schultz and Rudkin ('48) on "germ-free" Drosophila, make it apparent that not all animals possess the ability to synthesize niacin, and that the amount of tryptophan included in the diet in no way alters the situation.

TABLE 1

Standard supplements to the amino acid media
(All amounts are given in μg/m. of final medium.)

Dextrose	1000	Ca pantothenate	0.10
Sodium acetate	1000	Pyridoxine HCl	1.00
Tween 85[1]	700	Pyridoxal HCl	0.10
$MgSO_4 \cdot 7H_2O$	100	Pyridoxamine HCl	0.10
K_2HPO_4	100	Riboflavin	0.10
$CaCl_2 \cdot 2H_2O$	50	Pteroylglutamic acid	0.01
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	25	Biotin (free acid)	0.0005
$FeCl_3 \cdot 6H_2O$	1.25	Thiamine HCl	1.0
$CuCl_2 \cdot 2H_2O$	5	Choline chloride	1.0
$MnCl_2 \cdot 4H_2O$	0.50	Protogen[2]	0.375
$ZnCl_2$	0.05	Guanylic acid	30
		Adenylic acid	20
		Uracil	10
		Cytidylic acid	25

[1] Furnished by the Atlas Powder Co., Wilmington, Delaware.

[2] Supplied through the courtesy of Dr. E. L. R. Stokstad. For reference to this growth factor see Stokstad, Hoffman, Regan, Fordham and Jukes ('49) and Kidder and Dewey ('49a).

METHODS

The animal used in these studies was the ciliated protozoan, *Tetrahymena geleii,* strain W. This organism has been grown in pure (bacteria-free) culture in this laboratory for many years and its nutritional requirements are well known (Kidder and Dewey, '49a). In these investigations amino acids were used as the source of nitrogen, but these were varied to simulate some of the test diets used in similar studies on rats. This was done in order to obtain results which could be compared

with the same type of work on the higher animals. Thus 9%, 12%, 18% and 24% casein levels were simulated, the individual amino acids being added according to the published data on composition of casein (Hawk, Oser and Summerson, '47). All diets contained the various ingredients listed in table 1. Nicotinamide was used in varying concentrations, as will be indicated.

Cultures were incubated in a slanted position at 25°C. for 72 hours in 125 × 15 mm Pyrex test tubes and growth was measured turbidimetrically (Kidder and Dewey, '48a). Only the results of third serial transplants were considered. Experimental series were set up in triplicate and the individual tubes averaged for the final result. Each of the experiments was repeated varying numbers of times.

EXPERIMENTAL

Tetrahymena requires both tryptophan (Kidder and Dewey, '45, '48b) and niacin (Kidder and Dewey, '49b). If there is ability on the part of the organism to synthesize any niacin from tryptophan (or the reverse) then it should be possible to demonstrate sparing action, if not replacement. Accordingly, dose response experiments were conducted to determine the amount of nicotinamide required for half-maximum growth in the presence of increasing amounts of tryptophan. The amino acid levels for this set of experiments were the same as in simulated 12% casein (with tryptophan omitted). It was found that as the tryptophan was increased from a suboptimum amount (2 μg/ml) to a supraoptimum amount (28 μg/ml) there was a slight, but doubtfully significant, increase in the niacin requirement. There was certainly no sparing of niacin by high levels of tryptophan, which indicates clearly that Tetrahymena cannot synthesize the vitamin under these conditions.

In the light of reports regarding the effects of amino acids other than tryptophan on the apparent synthesis of niacin by the rat or the rat microflora (Ellinger and Kader, '47), an extensive series of experiments was conducted in which the

total amino acid concentrations, as well as the tryptophan levels, were varied. The nicotinamide requirement was tested under these conditions. The results are summarized in table 2. Although the total growth varied in relation to the total amino acid concentrations when these were used in the proportions

TABLE 2

Dose response to nicotinamide in varying concentrations of amino acids

(Values are given in optical density where no growth = zero)

NICOTINAMIDE	SIMULATED CASEIN LEVEL					
	12%	18%	24%	24% L-tryptophan (μg/ml)	12%	12%
	10	15	20	10	10 gelatin (12%)	10 glycine (2%)
μg/ml						
0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.005	0.018	0.002	0.101	0.070	0.001	0.029
0.006	0.039	0.035	0.135	0.115	0.039	0.060
0.007	0.072	0.054	0.162	0.179	0.055	0.093
0.008	0.080	0.090	0.196	0.188	0.086	0.117
0.009	0.100	0.115	0.222	0.211	0.132	0.159
0.010	0.139	0.154	0.247	0.235	0.166	0.181
0.025	0.233	0.299	0.324	0.325	0.236	0.250
0.050	0.251	0.345	0.352	0.358	0.248	0.261
0.100	0.260	0.342	0.354	0.361	0.249	0.259
Amount of nicotinamide for ½ max. growth (μg/ml)	0.0105	0.0105	0.008	0.008	0.009	0.008

characteristic of casein, the requirement for niacin was not appreciably different in any instance. It will be seen from the figures given in table 2 that the addition of 12% gelatin [2] or 2% gylcine neither increased total gowth nor decreased it. This last does not correspond to the results reported by Briggs, Luckey, Tepley, Elvehjem and Hart ('43) and Briggs ('45) on the chick, or with those obtained by Krehl, Sarma and El-

[2] Eastman ash-free.

vehjem ('46), Krehl, Henderson, de la Huerga and Elvehjem ('46) and Schweigert and Pearson ('48) with the rat, where depression of growth resulted which could be alleviated by added niacin or tryptophan.

Dose response experiments were conducted wherein the amount of tryptophan was varied in order to determine the effect of different levels of amino acids on the tryptophan requirement. Some of these results are summarized in table 3.

TABLE 3

Dose response to L-*tryptophan*

(Stimulated casein [minus tryptophan] at the 12% and 18% levels. Both media contain 0.01 μg/ml nicotinamide.)

L-TRYPTOPHAN	12% SIMULATED CASEIN LEVEL	18% SIMULATED CASEIN LEVEL
μg/ml		
0	0.000	0.000
2	0.105	0.057
4	0.218	0.230
6	0.260	0.294
8	0.269	0.309
10	0.261	0.342
12	0.255	0.339
14	0.260	0.321
25	0.237	0.332
50	0.209	0.310
Amount of tryptophan for ½ max. growth (μg/ml)	2.5	3.0

The amount of tryptophan required for half-maximum growth increased as the total amino acid levels increased.

Hankes, Henderson, Brickson and Elvehjem ('48) have reported that the addition of threonine or phenylalanine to a low casein diet causes severe growth retardation in rats. Protection is afforded by increased tryptophan levels. Similar experiments were conducted under our conditions and no growth inhibition resulted with increases of either amino acid.

Moreover, the results were the same when the tryptophan level was suboptimum (7.5 μg/ml) or high (27.5 μg/ml) and at low or optimum levels of nicotinamide (0.01 μg/ml and 0.1 μg/ml, respectively).

We have tested the effects of indole acetic acid in media containing varying amounts of tryptophan and nicotinamide. No inhibitory effects were found, as was earlier reported by Kodicek, Carpenter and Harris ('46). This was not unexpected in view of the general failure to confirm the earlier observation (Krehl, Carvalho and Cowgill, '47; Rosen and Perlzweig, '47; Kodicek, Carpenter and Harris, '47). Corn extracts, on the other hand, did produce growth inhibition. A corn extract prepared by us after the method of Woolley ('46) produced approximately 20% growth retardation when used at levels comparable with those used on mice. Essentially the same results were obtained with a corn extract of high "pellagragenic" potency which was kindly supplied to us by Dr. D. W. Woolley. In neither instance, however, was the inhibitory effect of the corn extracts counteracted by the addition of high levels of either nicotinamide or tryptophan or of both together. It appears, therefore, that growth of Tetrahymena is inhibited by some substance in corn, or perhaps by the materials used in the preparation of the extract, but the system or mechanism inhibited is not that concerned with tryptophan or niacin.

DISCUSSION

The results obtained in this study make it perfectly clear that the "germ-free" animal, Tetrahymena, has lost the enzymes necessary for the synthesis of niacin. It would appear, therefore, that this organism has lost an enzymatic mechanism retained by the mammal. When one considers the complications imposed on the investigator of mammalian biochemistry and nutrition by the intestinal microflora, and the possibilities of injected materials diffusing with the intestinal secretions into the alimentary tract where the microflora reside, it is not surprising that differences of opinion exist regarding the actual site of niacin synthesis. To our knowledge

only three "germ-free" animal species have been investigated with the niacin problem in mind. These are the chick embryo: the fruit fly, Drosophila; and the present organism, Tetrahymena.

Snell and Quarles ('41) reported a striking increase in niacin during embryonic growth in the chick. We have confirmed this observation. On the other hand, we were unable to confirm the report of Schweigert, German and Garber ('48) on the effect of injected tryptophan in increasing still further the niacin yield in chick embryos.[3] The fact exists at least that embryonic enzymes are present in the chick for niacin synthesis and the effect of added tryptophan appears unimportant. As yet the niacin precursor in the chick embryo is unknown.

Schultz and Rudkin ('48) report that "germ-free" Drosophila are entirely dependent upon an outside source of niacin and that their requirement is increased by the addition of high levels of tryptophan. Drosophila and Tetrahymena are thus similar with respect to niacin synthesis, although phylogenetically there is perhaps as great a difference between the insects and the protozoa as between the protozoa and the mammals.

The question of whether our results and those of Schultz and Rudkin ('48) merely reflect species variations from the vertebrates so far investigated, or whether animal potentialities for niacin synthesis are in reality more restricted than has sometimes been supposed, must await more widespread experimental use of "germ-free" animals of widely different varieties.[4]

SUMMARY

The ciliated protozoan, *Tetrahymena geleii* W, was grown in a chemically defined medium and tests were conducted to

[3] Unpublished results. Confirmation of Schweigert et al. ('48) has been given by Denton, Kellogg, Rowland and Bird ('49).

[4] Since this paper went to press Henderson and Hankes ('49) and Hundley ('49) have shown that the administration of tryptophan to the rat results in significant increases in niacin synthesis. Since the bacterial flora were largely removed by enterectomy, it appears that there can be little doubt that the site of a large portion of the niacin synthesis from tryptophan is in the mammalian tissue.

determine whether or not there is a relationship between tryptophan and nicotinamide synthesis in this organism. It was found that the level of tryptophan in the medium in no way affected the organism's inabilty to synthesize nicotinamide. Corn extracts, containing the "pellagragenic" agent, produced inhibition but the inhibition was not reversed by tryptophan or nicotinamide or both together. This "germ-free" animal, therefore, is similar to "germ-free" Drosophila in its requirement for exogenous niacin. The implications of these findings in relation to mammals are discussed.

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THE EFFECT OF ORAL ADMINISTRATION OF RUTIN ON BLOOD, LIVER AND ADRENAL ASCORBIC ACID AND ON LIVER AND ADRENAL CHOLESTEROL IN GUINEA PIGS[1]

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ONE FIGURE

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Since the discovery of *citrin* in Szent-Györgyi's laboratory (Armentano et al., '36), numerous authors have postulated a physiological relationship between the capillary permeability factor and vitamin C (Lindheimer et al., '42; Parrot et al., '45; Shanno, '46). In fact, it has been recommended that adequate ascorbic acid intake be ensured in therapeutic treatment with vitamin P preparations (Couch et al., '46). The study presented in this paper was undertaken in an attempt to see whether or not one of the "vitamin P" substances, namely rutin (the rhamnoglucoside of quercetin), might influence ascorbic acid levels in the organism of a species which requires a dietary source of vitamin C.

Previous work in this laboratory (Papageorge and Adair, '47) had failed to give conclusive evidence as to the possibility of a "sparing effect" by rutin on the plasma ascorbic acid of normal human subjects during a period of low C intake. It was decided to investigate this possibility further by use of experimental animals, the ascorbic intake of which could

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be controlled better than in volunteer subjects. Guinea pigs were, therefore, placed on adequate and on inadequate diets with respect to vitamin C and the effect of oral administration of rutin on ascorbic acid levels in blood, liver and adrenals was studied. Because of the suggestion that ascorbic acid and cholesterol are both associated with the formation and release of adrenocortical hormones (Sayers et al., '46), liver and adrenals were also analyzed for cholesterol in two of the three experiments. No blood cholesterol determinations were made since we had not observed any effect on serum cholesterol after ingestion of rutin in the preliminary work with human subjects (Papageorge and Adair, '47).

PROCEDURE AND METHODS

Young adult male guinea pigs, weighing initially 335 to 532 gm, were used. Three experiments were carried out, in each of which 8 animals were given rutin and 8 served as controls. The ascorbic acid intake was adequate in the first two experiments and inadequate in the third. Experiments 1 and 3 were terminated on the 22nd day and analyses were made for whole blood, liver and adrenal ascorbic acid, and for liver and adrenal cholesterol. Experiment 2 was designed to reproduce, under better controlled conditions, the significant findings of the first experiment. It was terminated on the 15th day and involved only blood and adrenal ascorbic acid determinations.

The basal diet was a commercial rabbit chow [2] supplemented weekly with oleo percomorphum. Water was given ad libitum. At the outset it was erroneously assumed that the ascorbic acid content of the chow was negligible, so that most of the animals in the first experiment were kept in pairs and food intakes were not recorded. Analysis of the chow not only proved our assumption wrong but showed also that the vitamin C content varied in different batches of chow even when this was exposed to the air as recommended by Sealock and

[2] Purina checkers, "complete ration."

Silberstein ('40) for destruction of ascorbic acid. Therefore, the animals of the second and third experimental groups were kept in individual cages, daily food intakes were recorded, and each batch of chow was analyzed for ascorbic acid. It was thus possible to arrive at a fairly accurate estimate of the total daily intake of ascorbic acid of each animal, as well as to achieve a more nearly uniform food intake than when two or more animals are kept together in the same cage.

Adequate ascorbic acid intake was ensured in the first two experiments by daily oral administration of 1.3 mg of the vitamin [3] per 100 gm body weight (Kuether et al., '44). The average total ascorbic acid intake (chow and supplement) was approximately 1.8 mg per 100 gm body weight per day in experiment 1. The corresponding intake in experiment 2 was calculated as 1.57 mg. No supplementary ascorbic acid was given to the guinea pigs in the third experiment; the daily vitamin C intake of the animals in this group averaged 0.40 gm per 100 gm body weight.

The rutin-fed guinea pigs in all three experiments were given a 20 mg tablet of rutin [4] by mouth every other day. On the day the experiment was terminated rutin was always given, but no food nor supplementary ascorbic acid.

At the end of the experimental period the animals were anaesthetized with nembutal, blood was taken by heart puncture, the organs to be analyzed were removed, and the analytical procedures were carried out. No more than 6 animals were placed on the test diet on a given day, so that the initiation and termination of each of the three experiments were staggered over a period of days in order to make possible completion of the chemical analyses within the required time. Oxalated whole blood was used for ascorbic acid determination. The liver samples and the adrenals were dissected quickly, weighed in 6% trichloraceteic acid on an analytical balance to the nearest 0.1 mg, and ground in the appropriate extracting solution (trichloracetic acid for vitamin C and

[3] Lilly's Cevalin.

[4] Sharp and Dohme, Inc.

acetone-alcohol for cholesterol) with a small tissue grinder. The left adrenal gland was used for ascorbic acid determination and the right for cholesterol except where only the former was determined, in which case both adrenals were used for ascorbic acid. Total ascorbic acid was determined by the technique of Roe and Kuether ('43), and the total cholesterol by Sperry's modification ('45) of the Schoenheimer and Sperry method. The analytical values were calculated as milligrams per 100 ml of whole blood or per 100 gm of fresh tissue.

RESULTS

Figure 1 presents the arithmetic means of the analytical findings. No appreciable difference was found between the rutin-fed animals and their respective controls in blood and liver ascorbic acid levels or in liver and adrenal cholesterol. The rutin-fed guinea pigs in group 1 (1.8 mg ascorbic acid per 100 gm body weight per day) showed a 13.1% greater mean level for adrenal cholesterol than their controls, but the absolute difference was very small and the individual variations were appreciable. The standard deviations from the mean values of adrenal cholesterol for group 1 were relatively great: 4.15 ± 0.71 for the rutin-fed and 3.67 ± 0.76 for the controls. Therefore, the difference of 0.48 mg % is not to be regarded as significant.

The most marked differences between rutin-fed and control animals were found in the adrenal ascorbic acid values, particularly in the first two groups, which were maintained on adequate vitamin C. In both of the adequate-C groups rutin appeared to increase the ascorbic acid concentration in the adrenals. This effect was not evident in the inadequate-C group, in which the rutin-fed animals showed a slightly lower adrenal ascorbic acid level.

Table 1 summarizes the statistical analysis of the data on adrenal ascorbic acid for all three groups. The standard

error of the difference between the means[5] indicates the same order of doubtful significance for the increase in the group 1 rutin-fed as for the decrease in the group 3 rutin-fed

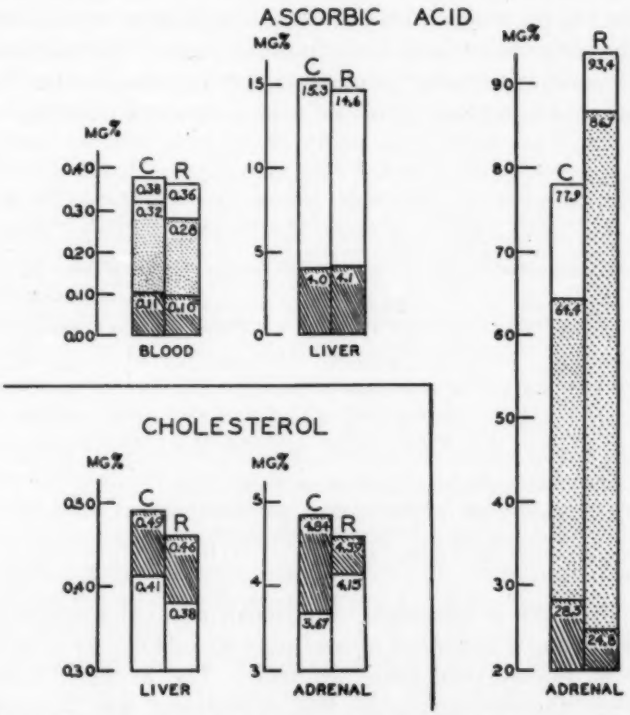

Fig. 1 Ascorbic acid and cholesterol levels in blood and tissues of three groups of guinea pigs maintained on different intakes of ascorbic acid. Mean daily ascorbic acid intake per 100 gm body weight represented as follows: blank areas = 1.8 mg for 21 days (group 1); dotted areas = 1.57 mg for 14 days (group 2); shaded areas = 0.40 mg for 21 days (group 3). C columns = control values; R columns = values for rutin-fed animals.

[5] The standard error of the difference between the means was calculated from the equation for small samples:

$$S_D = \frac{\sqrt{\frac{\Sigma(x^2_1) + \Sigma(x^2_2)}{(N_1 - 1) + (N_2 - 1)}}}{\sqrt{\frac{N_1 N_2}{N_1 + N_2}}}$$

animals. However, the absolute difference between the control and the rutin-fed mean values is relatively small in group 3. Furthermore, the individual variations in food intake and, therefore, in ascorbic acid intake were undoubtedly much greater in the first experiment than in the other two, in which the animals were kept in individual cages. The results of the second experiment supported our conclusion that rutin does increase adrenal ascorbic acid concentration when vitamin C intake is adequate. The mean adrenal ascorbic acid of the group 2 rutin-fed guinea pigs was 34.6% greater than that of the corresponding controls. The actual difference between the two means in this experiment was 22.3 mg %, and since this value is more than three times the standard error of the difference between the means, it is statistically significant.

TABLE 1

Differences between mean ascorbic acid concentrations of rutin-fed and control guinea pigs on adequate and inadequate vitamin C intakes

GROUP	VITAMIN C INTAKE		ASCORBIC ACID CONCENTRATION IN ADRENALS				
	Mean per day [1]	No. days [2]	Control means	Rutin-fed means	Increase on Rutin	Difference between means	S_D [3]
	mg/100 gm		*mg %*	*mg %*	*%*	*mg %*	
1	1.8	21	77.9 ± 16.40 [4]	93.4 ± 15.43	19.9	15.5	± 8.25
2	1.57	14	64.4 ± 13.56	86.7 ± 13.15	34.6	22.3	± 6.91
3	0.40	21	28.3 ± 3.76	24.8 ± 3.06	−12.4	3.5	± 1.71

[1] Milligrams of ascorbic acid per 100 gm body weight.
[2] Number of days on indicated intake before analysis.
[3] Standard error of the difference between means.
[4] Standard deviation of mean.

DISCUSSION

The concentrations of blood and liver ascorbic acid in all three groups, both rutin-fed and control animals, are in general agreement with those reported by Kuether, Telford and Roe ('44) for guinea pigs maintained on similar levels of vitamin C but for longer periods of time. The liver con-

centrations found by us are somewhat higher but the difference can hardly be regarded as significant in view of the considerable individual variations encountered in such work. The group 3 animals, maintained on an average daily intake of 0.40 mg of ascorbic acid per 100 gm body weight, had a mean adrenal ascorbic acid value of the same order as that found by Kuether and his co-workers with a corresponding intake of 0.701 mg. However, our controls on adequate C intake had adrenal ascorbic acid levels which compare more closely with those reported by these authors for similar ascorbic acid intakes. This makes more likely our belief that the increase in adrenal ascorbic acid of the rutin-fed animals on adequate ascorbic acid intakes was actually due to the administration of rutin.

Our findings are only in partial agreement with those of Cotereau et al. ('48), who recently reported a "sparing effect" of a "vitamin P" substance on liver, spleen, kidney and adrenal ascorbic acid in the guinea pig. Cotereau and his co-workers found 4 to 8 times more vitamin C in these organs when 3 mg of catechin as well as 10 mg of ascorbic acid were added daily to the basic diet than when ascorbic acid alone was added. Catechin (3, 5, 7, 3′, 4′ pentahydroxy-phenyldihydrochromane) without supplementary vitamin C had no effect on the vitamin C levels of the scorbutic animals nor on their survival time.

Our results neither support nor contradict the suggestion that "vitamin P" is necessary for the absorption and retention of ascorbic acid (Elmby and Warburg, '37; Warter et al., '48); nor do they provide evidence in favor of the theory that one function of "vitamin P" is to lower the need for ascorbic acid (Parrot et al., '45). In the vitamin C-inadequate group we observed no difference in rate of gain of body weight nor in general well-being between the controls and the rutin-fed guinea pigs. Furthermore, if the whole blood level of ascorbic acid reflects its concentration in the tissues and is, therefore, a significant indication of nutritional status with respect to this vitamin (Kuether et al., '44), then rutin

does not appear to lower the need for ascorbic acid, at least under the conditions of our experiments.

The influence of rutin in raising the vitamin C concentration in the adrenal when adequate supplies of the vitamin are available may be the result of its antioxidant effect with respect to ascorbic acid (Rayle and Papageorge, '48) and also toward epinephrine (Lavollay, '41; De Eds, '47), since oxidation products of the hormone markedly accelerate oxidation of ascorbic acid in the presence of cytochrome C (Hermann et al., '46). Kuether and his co-workers ('44) showed that as ascorbic acid intake was increased above the level necessary to prevent any deficiency symptoms (1.23 mg per 100 gm of body weight per day), the concentration in the adrenal rose in greater proportion than in blood, brain muscle, kidney, spleen, heart or liver. It is thus probable that when amounts of ascorbic acid in excess of the minimum necessary to meet metabolic requirements are available, rutin prevents destruction of some of the surplus vitamin through its antioxidant effect and thus permits increased deposition of ascorbic acid in the organ which has the greatest avidity for storing it.

SUMMARY

1. The influence of oral administration of rutin on total ascorbic acid concentrations of whole blood, liver and adrenals, and on total cholesterol of liver and adrenals, was studied in two groups of guinea pigs, one of which was maintained on adequate and the other on inadequate levels of vitamin C intake for three weeks. Sixteen animals were used in each group, of which 8 served as controls and 8 were given 20 mg of rutin every other day throughout the test period. A third group of guinea pigs was maintained on an adequate ascorbic acid intake for only two weeks, and only the blood and adrenal vitamin C concentrations of this group were determined.

2. No significant differences in mean values between the rutin-fed animals and their respective controls were found except in the adrenal ascorbic acid concentrations of the two

groups on adequate vitamin C intake, which were as follows: (1) 77.9 gm % for controls and 93.4 mg % for rutin-fed; (2) 64.4 mg % for controls and 86.7 mg % for rutin-fed.

3. This "sparing effect" of rutin on adrenal ascorbic acid under conditions of adequate vitamin C intake may be due to the antioxidant action of rutin with respect to ascorbic acid and epinephrine, which latter when oxidized contributes to the destruction of vitamin C. Rutin may thus protect some of the surplus ascorbic acid from oxidation and permit increased deposition of the vitamin in the adrenals.

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